From genetic disease to protein structure: Analysis of disease associated missense mutations of the ABCC6 transporter

PhD thesis

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TABLE OF CONTENTS

I. ABBREVIATIONS

4-PBA:	4-phenylbutirate
5mC:	5-methyl cytosine
ABC:	ATP-Binding Cassette
AGE:	Advanced glycation end product
ALP:	Alkaline phosphatase
AMD:	Age-related macular degeneration
AMP:	Adenosine monophosphate
ANK:	Ankylosis protein
ATP:	Adenosine triphophate
BM:	Bruch's membrane:
BMP:	Bone morphogenic protein
BRB:	Blood-retina-barrier
BSA:	Bovine serum albumin
C:	Cytosine
C/EBP:	CCAAT/enhancer binding protein
CAD:	Coronary artery disease
CALJA:	Calcification of joints and arteries
CaR:	Ca sensing receptor
CFTR:	Cystic fibrosis transmembrane conductance regulator
CGI:	CpG island
CHD:	Coronary heart disease
CHO:	Chinese hamster ovary
CM:	Chylomicrone
CR:	Chylomicrone remnant
DAPI:	4',6-diamino-2-phenylindole
DMEM:	Dulbecco's Modified Eagle Medium
DNP-SG:	S-(2,4 dinitrophenyl) glutathione
DOC:	Deoxycholate
DPBS:	Dulbecco's modified PBS
DTT:	1,4-dithiotreitol
E217βG:	17-β-estradiol-17-β-D-glucuronide
ECL:	Enhanced chemiluminescence
EDTA:	Ethylene diemine tetraacetic acid
EGTA:	Ethylene glycol tetraacetic acid
ENPP1:	Nucleotide pyrophopsphate/phosphodiesterase 1 enzyme
ERK1/2:	Extracellular signal-regulated kinase 1/2
ESRD:	End stage renal disease
EURORDIS:	The Europian Organisation for Rare Diseases
FBS:	Fetal bovine serum
FDA:	Food and Drug Administration
FGF-23:	Fibroblast growth factor
G:	Guanine
GACI:	Generalized arterial calcification of infancy
GAG:	Glucose amino glycans

GALV:	Gibbon ape leukemia virus envelope glycoprotein
GGCX:	Gamma-glutamyl carboxylase
Gla-MGP:	Uncarboxylated form of MGP
Glu-MGP:	Fully maturated γ–carboxylated MGP
GSH:	Glutathione
HA:	Hydroxyapatite
HEK-293:	Human embryonic kidney cell line
HEPES:	4-(2-hydroxyethyl)piperazin-1-ethanesulfonic acid
HGVS:	Human Genome Variation Society
HlyB:	Hemolysin B
HPLC-MS:	High pressure liquid chromatography-mass spectometry
HPLC:	High pressure liquid chromatography
HRP:	Horseradish peroxidase
IBGC:	Idiopathic basal ganglia calcification
ICL:	Intracellular loop
IM:	Indomethacine
IUPAC:	International Union of Pure and Applied Chemistry
K3:	Vitamin K3, menadione
K3GS:	Menadione-GSH conjugate
KH2:	Reduced Vitamin K
K_M:	Michaelis constans
KO:	Vitamin K-epoxide
LOVD:	Leiden Open (source) Variation Database
LTC4:	Leukotriene C4
MAM:	Mitochondria associated membrane
MAPK:	Mitogen-activated protein kinase
MDCKII:	Madin-Darby canine kidney cell line II
MGP:	Matrix Gla protein
MK:	Menaquinone
MMP:	Matrix metalloproteinase
MRP:	Multidrug resistance associated protein
MTX:	Methotrexate
NBD:	Nucleotide binding domain
NCATS:	National Center for Advancing Translational Sciences
NCBI:	National Center for Biotechnology Information
NEM-GS:	N-ethylmaleimid S-glutathione
NFkB:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH:	National Institute of Health
NORD:	National Organization for Rare Disorders
nsSNP:	nonsynonymous single-nucleotide polymorphism
NT5E:	5'-ectonucleotidase (CD73)
OMIM:	Online Mendelian Inheritance in Man
OPN:	Osteopontin
P-gp:	P-glycoprotein
PBS:	Phosphate Buffered Saline
PDB ID:	Protein Data Bank identification code
Phenodex:	PXE phenotype index
Pi:	phosphate

PiT-2:	Type III sodium-dependent Pi co-transporter
PMSF:	Phenylmethylsulfonyl fluoride
PPi:	Pyrophosphate
PTH:	Parathyroid hormone
PVDF:	Polyvinylidene difluoride
PXE:	Pseudoxanthoma elasticum
RAGE:	Receptor for advanced glycation end products
RAMEB:	Randomly methylated-beta-cyclodextrin
SDS:	Sodium dodecyl sulfate
Sf9:	Spodoptera frugiperda ovarian cells
SMC:	Smooth muscle cell
STAT3:	Signal transducer and activator of transcription 3
TMD:	Transmembrane domain
TMEP:	Tris Mannitol EGTA buffer
TNAP:	Tissue nonspecific alkaline phosphatase
TRL:	Triglyceride-rich lipoproteine
Ttw mice:	Tiptoe walking mice
VDR:	Vitamin D receptor
VEGF:	Vascular endothelial growth factor
VitK:	Vitamin K
VK3GS:	Menadione-GSH conjugate
VKORC1:	Vitamin K 2,3 epoxide reductase
V_{max}:	Maximal value of initial velocity
VSMC:	Vasculas smooth muscle cell
WHO:	World Health Organization

II. INTRODUCTION

This thesis concludes my contribution to our scientific knowledge about the molecular background of a rare genetic disease, pseudoxanthoma elasticum (PXE), and the ABCC6 protein encoded by the disease gene.

II.1. Basic functional and structural properties of human ABC transporters

Human ABC (ATP Binding Cassette) proteins are ATP utilizing transmembrane transporters that pumps substrates across the plasmamembrane and intracellular membranes. ABCs within eukaryote cells are typically exporters, drive transport out of the cytoplasm or accumulate compounds within organelles. Most of them are classical transporters with a few exceptions of receptors and channel proteins. The catalytic nucleotide binding domains (NBDs or ABCs) face the intracellular space of the cell, see Fig.1. Transmembrane domains (TMDs) form a substrate translocation pathway, see Fig.2b, that is connected to ABC domains via intracellular helices, rigid extensions of the membrane embedded helices. A functional core unit of an ABC transporter consists of two NBDs and two TMDs. ABC genes are organized as either full transporters, coding for the entire functional core, or as half transporters containing one of each domain. The latter proteins form either homodimers or heterodimers to constitute a functional transporter [Dean et al, 2001]. Proteins are classified as ABC proteins due to the sequence and organisation of the ATP-binding domains that harbour Walker-A and Walker-B motifs, found in most of the ATP-binding proteins [Dean et al, 2001], and an additional element, the signature motif (C-motif), characteristic to ABC transporters, see Fig23. [Hyde et al, 1990].

ABC genes can be found in all organisms. They are abundant in eukaryotic organisms and are highly conserved between species, indicating that most of them existed since the beginning of eukaryotic evolution [Dean et al, 2001]. The human genome codes for 48 ABC transporters that are grouped into seven subfamilies from ABCA to G. ABCC6 protein belongs to the C subfamily that harbours twelve members; all of them are all full transporters. The proximate relative of ABCC6 is ABCC1; amino acid identity of the two proteins is 45%.

ABCC1, ABCC2, ABCC3 and ABCC6 form a subgroup of ABCC type transporters, the long type ABCCs (older name: long MRPs), in which the core ABC structure of two nucleotide-binding and two transmembrane domains is extended with an additional transmembrane domain, TMD0, through a flexible linker, L0. We have very limited information about the role of these extra domains. ABCC1 protein was shown to retain only 10% of its transport activity in the absence of the whole TMD0 and L0 region if expressed and measured in Sf9 insect cells [Bakos et al, 1998]. If analyzed in polarized MDCKII cells the truncated protein, ΔABCC1, lacking TMD0 and L0 was not targeted to the cell surface. Co-expression of the L0 linker with the ΔABCC1 rescued the protein, and it was targeted to the basolateral plasmamembrane, like the wild type. Interestingly, the L0ΔABCC1 construct also behaved like wild type protein in functional measurements, indicating that the L0 but not the TMD0 domain is necessary for localization and function of ABCC1 *in vitro*. In contrast to L0ΔABCC1, L0ΔABCC2 was retained in intracellular compartments if expressed in polarized MDCKII. Co-expression with TMD0 resulted in wild type like apical localization and transport activity [Fernández et al, 2002]. Authors concluded that TMD0 region is required for function, routing, and stable association to the apical membrane of ABCC2.

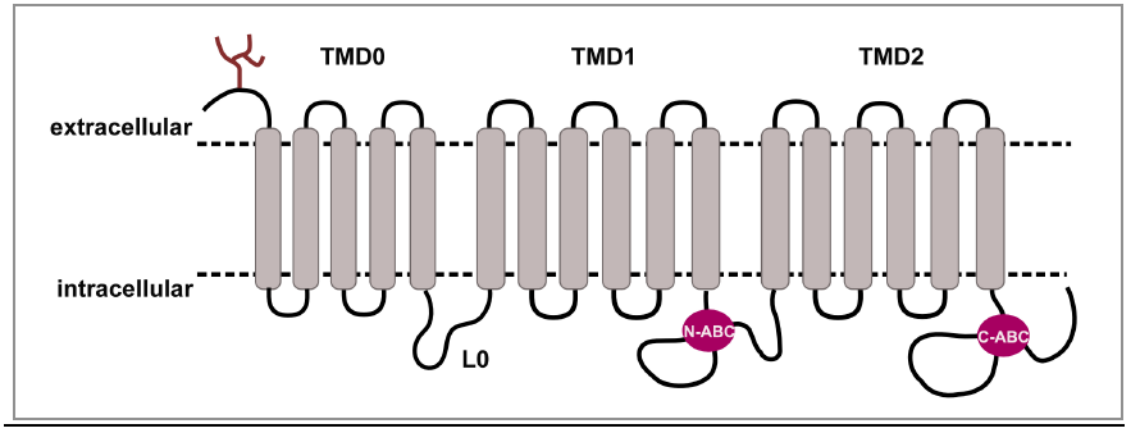

Fig.1: Schematic representation of the membrane topology of the long type ABCC transporters. The core ABC structure of two nucleotide-binding and two transmembrane domains is extended with an additional transmembrane domain, TMD0 through a flexible linker, L0. Image was created by László Barna.

The ABC domains are organised in a head to tail orientation to form two composite catalytic sites that close upon ATP binding. The two nucleotide-binding pockets are formed by Walker-A and Walker-B motifs of one nucleotide binding fold and by the

signature motif of the opposite nucleotide-binding fold, see schematic representation on Fig.2a. Models built on crystallisation data revealed several features of the structural organisation of these large transmembrane proteins, however structural transitions upon function remains a great challenge for researchers.

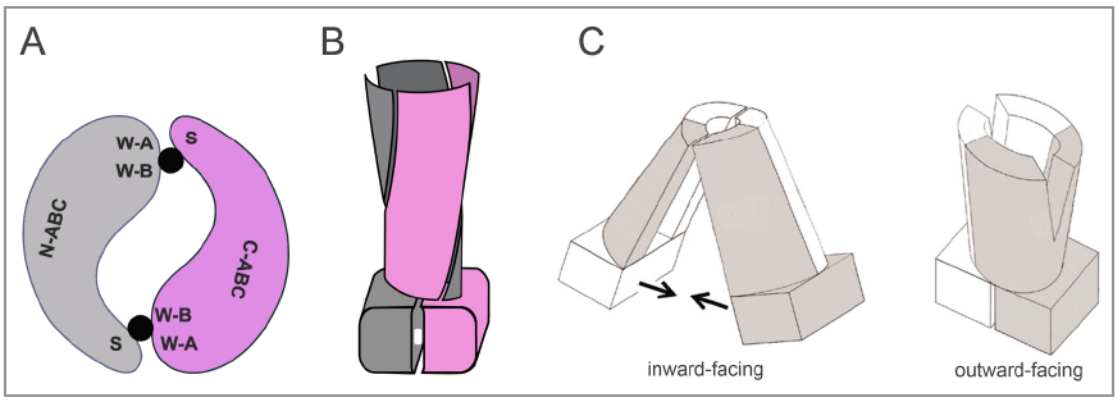

Fig.2: Schematic representation of orientation of nucleotide binding and transmembrane domains during catalytic and transport function of ABC transporters. A: head to tail orientation of the N- and C-terminal ABC domains forming the two hybrid catalytic sites. ATP is symbolised with black dots. W-A, W-B and S abbreviations indicate the Walker-A, Walker-B and the Signature motifs, respectively. B: schematic representation of a full length ABC transporter, the N-and C-terminal parts of the protein are coloured in grey and pink, respectively. Domain swapping of TMD1 and TMD2 and the two nucleotide binding domain is also visible on this view. C: This cartoon represents the two end point conformations related to the transport mechanism of an ABC transporter. Figures on panel A and B were created by László Barna, figures of panel C were adopted from Ward et al, 2007.

Transmembrane domains that form the substrate translocation pore are connected to the catalytic NBDs through rigid helices that transmit conformational changes generalized during the ATP-cycle. The short coupling helices within these structures of the TMD1 and TMD2 (two in each) directly contact both nucleotide-binding folds. This special type of domain swapping, see Fig.2b, was first described by Dawson et al analysing the structure of the Sav1866 [Dawson et al., 2006]. The two endpoints of the structural transitions during a functional cycle of substrate translocation, the ‘inward facing’ and the ‘outward facing’ conformations are illustrated on Fig.2c. Since we do not have any crystal structure data of the TMD0 and L0 regions these domains cannot be included in the models. To date (30.03.2014) there are the following crystal structure coordinates of full ABC transporters available: Sav1866 bacterial transporter (outward-facing conformation; PDB ID: 2ONJ; 3.4Å) [Dawson et al, 2006 and 2007]; mouse Abcb1 (inward-facing conformation; PDB

ID: 3G5U; 3.8Å) [Aller et al., 2009]; mouse Abcb1 revised, inward-facing; PDB ID: 4M1M; 3.8Å) [Li et al, 2013]; C. elegans P-gp/Abcb1 (inward-facing conformation; PDB ID: 4F4C; 3.4Å) [Jin et al, 2012].

II.2. ABC transporters related to human genetic diseases

ABC proteins transport a wide spectrum of endogenous and xenobiotic compounds within the human body. Some of them are highly specialized, while others are promiscuous transporters. Several ABC transporters are typically expressed on barrier surfaces of the body, like the blood-brain barrier, blood-placental barrier, blood-testis barrier, where they protect these organs from toxic metabolites. Others are involved in secretory mechanisms in different structures, e.g. the hepatic surface of bile canaliculi, excretory ducts of mammary glands, tubular epithel in kidney, respiratory epithel. Mutations in ABC genes were proved as the genetic basis of several single gene Mendelian diseases [Klein et al, 1999], see table1, typically recessive disorders. Heterozygous variants of several other ABCs are associated to increased susceptibility to multifactorial diseases.

transporter	Mendelian disease	OMIM#
ABCA1	Tangier disease; Hypoalphalipoproteinemia	205400; 604091
ABCA3	Surfactant metabolism dysfunction, pulmonary,3,SMDP3	610921
ABCA4	Stargardt disease	248200
ABCA12	Harlequin ichtyosis Ichthyosis congenita IIB	242500 601277
ABCB2 (TAP1)	Bare lymphocyte syndrome	604571
ABCB3 (TAP2)	Bare lymphocyte syndrome	604571
ABCB4	Progressive familial intrahepatic cholestasis-3	602347
ABCB7	X-linked sideroblastosis and anemia	
ABCB11	Progressive familial intrahepatic cholestasis-2	601847
ABCC2	Dubin-Johnson	237500
ABCC6	Pseudoxanthoma elasticum; Generalized arterial calcification of infancy	264800 208000
ABCC7 (CFTR)	Cystic Fibrosis	219700
ABCC8 (SUR1)	Familial persistent hyperinsulinemic hypoglycemia of infancy	256450
ABCC9	Cardiaomyopathy, dilated, 10	608569
ABCD1	Adrenoleukodystrophy	300100
ABCG5	Sitosterolemia	210250
ABCG8	Sitosterolemia	210250

Table 1: Single gene Mendelian disorders associated to mutations of *ABC* genes.

II.2.1. Mutation within the* ABCC6 *gene are the genetic bases of two heritable disorders related to soft tissue calcification: pseudoxanthoma elasticum (PXE) and generalized arterial calcification of infancy (GACI).

In 2010 mutations within the *ABCC6* gene, previously thought of as the gene exclusively related to pseudoxanthoma elasticum (PXE), were demonstrated to be also causative in generalized arterial calcification of infancy (GACI) [Le Boulanger et al, 2010]. Moreover, mutations within the *ENPP1* gene, the diseases gene of GACI were also clearly shown to mimic PXE phenotype [Nitschke et al, 2012].

II.2.1.a Pseudoxanthoma elasticum (PXE; OMIM: 264800) is a recessive genetic disorder characterized by progressive calcification of elastic fibers in the skin, in the medial layer of middle-sized arteries and within the Bruch's membrane of the retina. The disease has been first identified in the late 1800's by French dermatologists. In 1896 the name "pseudo-xantome élastique" was given by Darier differentiating the skin lesions in PXE from xanthomas [Grimmer, 1961; Uitto 2014]. Grönblad and Strandberg a Swedish ophthalmologist and a dermatologist discovered the association between the cutaneous and ophthalmologic findings, giving the name: Gröndlad-Strandberg syndrome [Donaldson, 1983; Strandberg, 1929; Grönblad, 1929; Uitto 2014]. The cardiovascular symptoms were identified several decades later by Carlborg [Carlborg, 1944]. Finally, in 2000, the gene, responsible for the development of this multisystem orphan disease, was identified by positional cloning in three research groups [Bergen et al, 2000; LeSaux et al, 2000; Struck et al, 2000; Ringpfeil et al, 2000]. Experiments revealed mutations within the *ABCC6* gene as the genetic basis of PXE.

The ABCC6 transporter has been extensively studied since than. Tissue distribution and subcellular localization studies revealed marked liver expression with exclusive basolateral localization and with no or extremely low within tissues affected by the disease (also described later), suggesting a role for ABCC6 in the sinusoidal efflux of an unidentified metabolite. According to this PXE was hypothesized as a metabolic disorder [Jiang et al, 2009]. The physiologic substrate of ABCC6 and the pathological background of the disease still remain unknown.

ABCC6 -/- mice recapitulate features of pseudoxanthoma elasticum, they develop ectopic calcification of several tissues including the skin, arterial blood vessels, the retina, and the vibrissae [Klement et al, 2005; Gorgels et al, 2005]. The *Abcc6* locus was also

associated to the Dyscalc1 phenotype in mice [Meng et al, 2007] that is characteristic to dystrophic calcinosis at sites of inflammation and necrosis [Wang et al, 2009].

The estimated prevalence of PXE disease is 1 in 50.000 that would suggest approximately 150.000 patients affected worldwide. According to the high phenotypic variability in the manifestation of the disease this number may be an underestimate [Uitto 2014]. The basis of this variability remains currently unknown. Milder forms of the disease often remain undiagnosed even for lifetime [Neldner, 1988]. Most severe clinical consequences of the disease are the risk of cardiovascular events and the macular degeneration leading to loss of visual acuity. The first cutaneous findings are small lesions, yellowish papules that develop at characteristic sites of the skin, predominantly on predilection sites, like the lateral neck, antecubital and popliteal fossae [Neldner etal, 1998]. In later stages papules coalesce into larger plaques and the skin loses its elasticity, see Fig.3a,b,c. Skin manifestations are the first diagnostic signs, however they usually remain unrecognized until the second or third decade of life until the development of ocular and vascular complications. The earliest ocular symptom is the presence of peau d'orange sign of the fundus [Georgalas et al, 2009]. In later stages angioid streaks develop (neovascularization), see Fig.3d,e, that is most probably the result of the mineralization and subsequent fragmentation of the retinal Bruch's membrane. This may lead to complete central vision loss. The clinical signs of the cardiovascular symptoms are consequences of calcification affecting the lamina media in arterial vessel walls, also associated with intimal hyperplasia. These include loss of peripheral pulse, intermittent claudication, renovascular hypertension, gastrointestinal hemorrhage, intestinal angina, coronary artery disease with angina pectoris and in rare cases myocardial infarction [Uitto, 2014].

The diagnostic criteria include the characteristic hystopathologic finding of hydroxyapatite deposition within elastic fibers detected in skin biopsy samples with Hematoxylin-eosin staining combined with Verhoeff-Gieson and Von Kossa for elastin and calcium deposits, respectively; fundoscopy for angioid streaks, peau d'orange, macular degeneration; and mutational analysis of *ABCC6* gene. Diagnostic criteria of phenotypic stages were standardized in 2007, the system is called Phenodex (PXE phenotype index) [Pfendner et al, 2007].

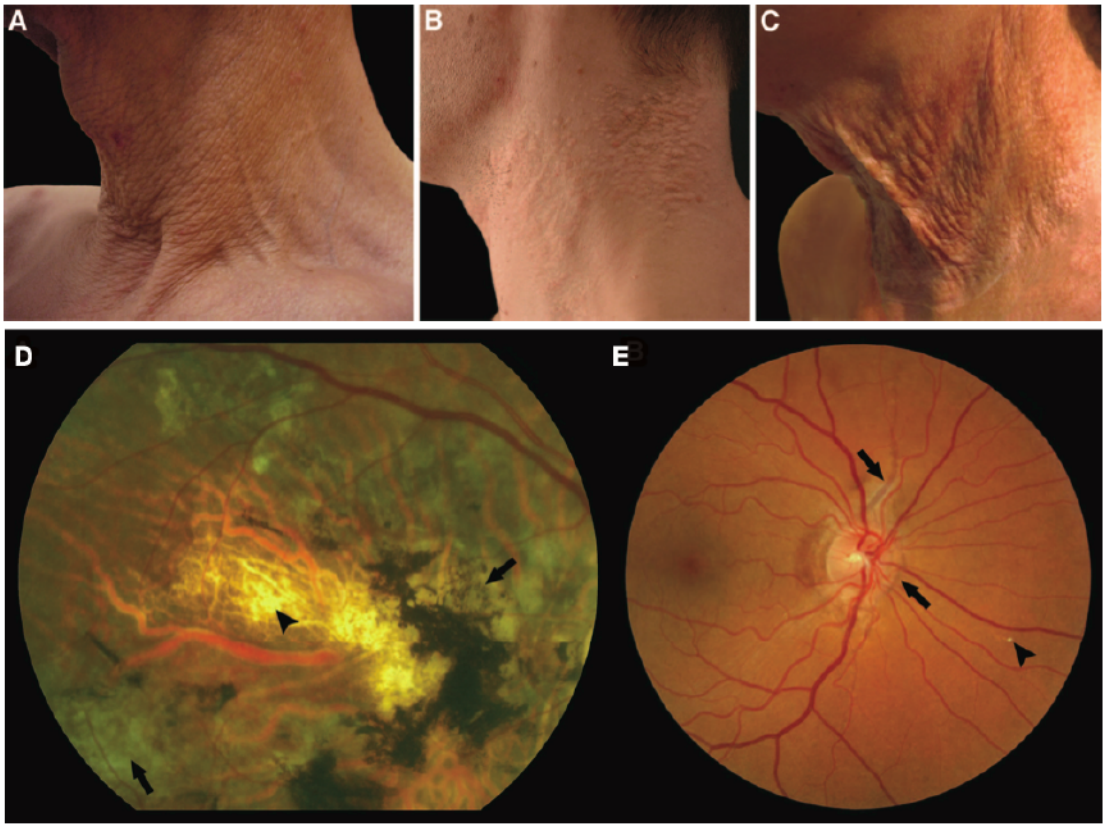

Fig.3: Characteristic papules, plaques and the loss of elasticity of the skin in PXE patients, panel A,B,C. Macular degeneration in PXE patients, panel D,E. 'Wet' type (arrows) and 'dry' type (arrowhead) macular degenerations are visible on panel D. Angioid streaks (arrow) and comet (arrowhead) are shown on panel E. These images were taken from [Plomp et al, 2009].

Standardized method for mutational detection within the *ABCC6* gene is concluded in [Pulkkinen et al., 2001]. Crucial points of mutational detection within *ABCC6* gene are the PCR primers, described in the latter paper, optimized for discriminating between the exons 1-9 of the *ABCC6* gene and those of the two pseudogenes. The gold standard of molecular diagnosis is identifying homozygosity or compound heterozygosity for known disease-causing mutations in the *ABCC6* gene. To date no PXE cases has been described having biallelic disease-causing mutation in *ABCC6* without clinical signs older than 30 years of age. All of these individuals have both ocular findings and skin lesions. Therefore two mutations within the gene would make the diagnosis of PXE, however in 10% of individuals with complete ophthalmologic/dermatologic diagnosis of PXE no or only one detectable mutation can be identified [Uitto, 2014]]. Standardization in both molecular and clinical diagnostic methods is necessary for the development of standardized clinical trials as well as in understanding genotype-phenotype correlations of the disease.

In PXE high variance in severity of symptoms can be observed between patients with the same mutations even within the same families, i.e. in almost identical genetic background. This indicates the modifying role of environmental factors or other genes. A number of acquired chronic and genetic diseases were reported to mimic some of the symptoms of PXE. These are β-thalassemia, sickle cell disease, chronic kidney disease, idiopathic hypercalcemia, hyperphosphatemia, familial tumoral calcinosis [Uitto, 2014]. Patients with a single gene Mendalian disorder, the PXE-like syndrome, underlined by mutations in the gamma-glutamyl carboxylase (GGCX) gene, also develop cutaneous signs similar to PXE. The GGCX enzyme is responsible for the secondary modification, thus the activation of Ca-chelating proteins. These include vitamin-K dependent coagulation factors and proteins participating in anti-calcification processes of connective tissues. PXE-like patients also develop blood clothing factor deficiency that is absent in PXE disease. The ABCC6 protein is expressed predominantly in the liver. It exports yet unidentified substrate(s) into the circulation. This suggested that while the defective GGCX enzyme could be responsible for the hepatic and peripheral manifestation of PXE-like syndrome the lack of vitamin-K compounds at peripheral tissues would be the basis of ABCC6 associated calcification processes in case of PXE. According to this hypothesis ABCC6 would be the transporter of (a) vitamin-K form or derivative utilized in peripheral tissues. Although these diseases mimic some of the calcification symptoms, predominantly the skin signs, none of these patients develop both skin and eye disorders, thus PXE can be distinguished from these based on the accurate diagnostic criteria.

II.2.1.b Generalized arterial calcification of infancy (GACI; OMIM: 208000) is a rare genetic disorder that was first described in 1901 [Bryant and White, 1901]. Patients develop severe calcification of the lamina media of large and medium sized arteries that is associated with the proliferation of the intimal layer, see Fig.4, leading to stenosis of the vessels and myocardial ischemia within the first months of life. The diagnosis is often made by prenatal ultrasound. Although survival until adulthood has been reported most of the patients die within the first 6 month of life due heart failure [Nitschke et al, 2012].

The low levels of PPi in the serum and in the arterial biopsy of an affected proband with deficiency of nucleotide pyrophopsphate/phosphodiesterase 1 (NPP1) enzyme lead to the discovery of mutations in *ENPP1* gene (coding for NPP1 enzyme) in GACI patients

[Rutsch et al, 2000]. Most of the patients with classical GACI symptoms were found to carry biallelic mutations of the *ENPP1* gene [Rutsch et al, 2003; Ruf et al, 2005; Rutsch et al, 2008]. NPP proteins are highly conserved between species, they have wide substrate specificity. NPP family consists of 5 members in human. Primary function of NPP1 is the generation of ectopic pyrophosphate (PPi) [Rutsch et al, 2000], a potent inhibitor of hydroxiapatite deposition, by hydrolyzing ATP. NPP1 is a 104kDa homodimeric membrane spanning protein with a large catalytic extracellular domain that can be cleaved and it may function as a secreted circulating protein. ENPP1 is widely expressed, including the cartilage, heart, kidney, parathyroid and skeletal muscle, and it is also abundant in vascular smooth muscle cells, osteoblasts and chondrocytes [Mackenzie et al, 2012].

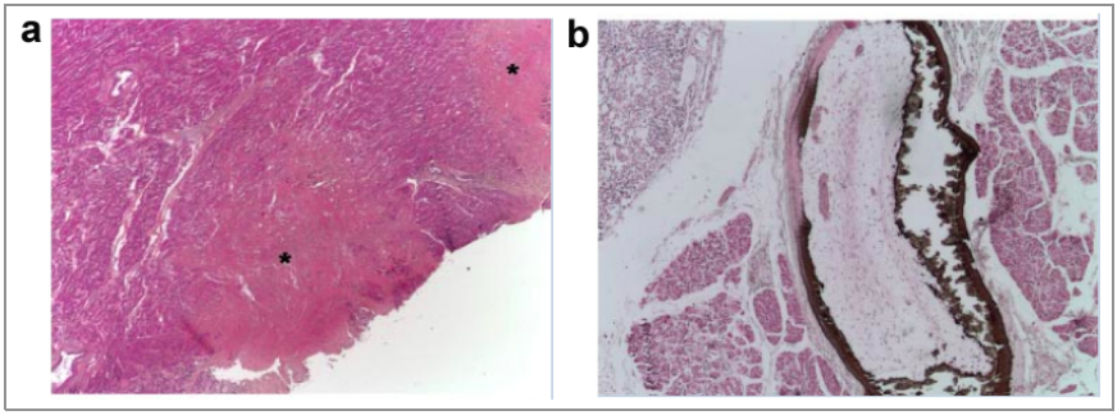

Fig.4: Myocardial (a) and pancreatic artery (b) tissue sections from patients with generalized arterial calcification of infancy. a: Hematoxylin-eosine staining of myocardial tissue, 50x magnification, regions of myocardial infarction are indicated by stars. B: Von Kossa staining of a pancreatic artery, 200x magnification, brown staining indicates massive wall calcification. Intimal proliferation is visible within the brown area. This figure was taken from Le Boulanger et al, 2009.

Rodent models of GACI disease: the tiptoe walking (*ttw/ttw*) mice [Okawa et al, 1998], carrying truncating mutation of *Enpp1* gene, and the transgenically engineered *Enpp1-/-* mice [Sali et al, 1999] recapitulate the symptoms of classical GACI disease.

Cardiovascular alterations in patient with homogeneous genetic background and similar sonographic and radiographic features show large variability even within one family [Nitschke et al, 2012] that suggest important role for additional genetic factors that may influence disease outcome. In 2010 a family with two brothers was described by Le Boulanger et al. Elder brother had uncomplicated pseudoxanthoma elasticum, with mutations in the *ABCC6* gene; while the younger brother died of a sever condition similar to that of found in GACI patients. Unfortunately no DNA of this infant was available. No

ENPP1 mutation could be identified in any living members of the family [Le Boulanger et al, 2010]. Interestingly, two missense mutations of the *ABCC6* gene (p.R765Q and p.Q1406K) have been identified in the DNA sample of the elder brother, each mutation was inherited from one of his asymptomatic parents. The p.R765Q mutation was previously described from DNA samples of several PXE patients, while the p.Q1406K mutation was new. Further studies revealed 14 additional GACI cases with *ABCC6* mutations in which no *ENPP1* mutation could be identified previously. These ABCC6 mutations were in some of the cases novel but in some individuals they identified PXE-associated mutations that were associated with relatively mild, slow progressing pathology in PXE. The severe and early onset of GACI disease is in marked contrast with the typical late manifestation of PXE and the relative normal lifespan of the PXE patients. Interestingly Nitschke et al also found 3 GACI patients with *ENPP1* mutations who developed characteristic symptoms of PXE between 5 and 8 years of age. No *ABCC6* mutations could be identified in DNA samples of these patients, however clinical findings are indistinguishable from classical PXE patients with mutations in the *ABCC6* gene [Nitschke et al, 2012], see Fig.5.

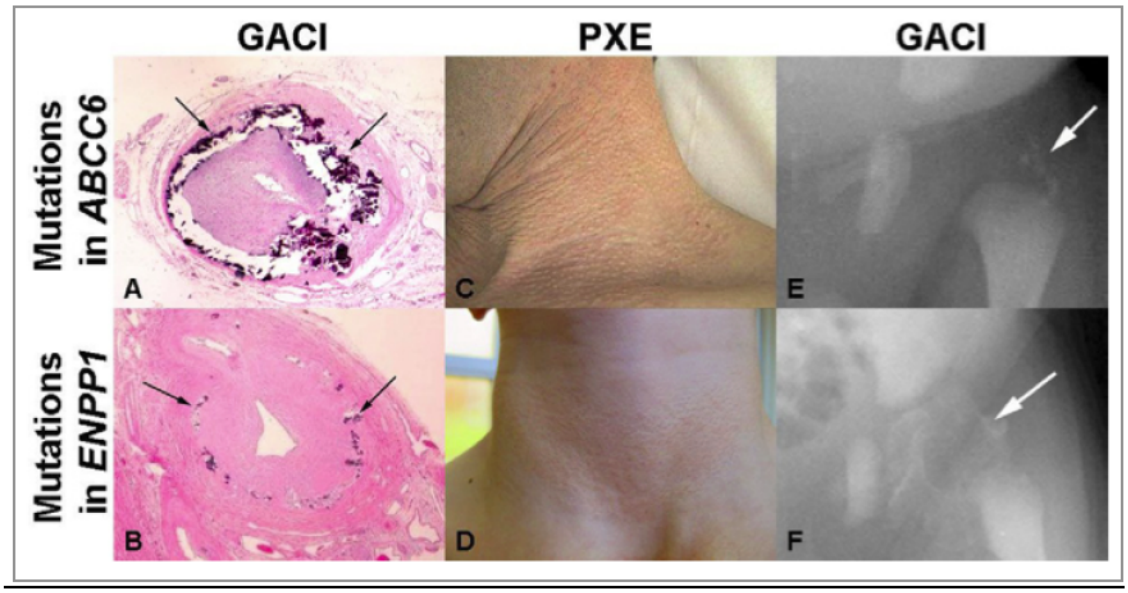

Fig.5: Genocopy of symptoms observed in patients with ABCC6 or ENPP1 mutations in GACI and PXE diseases. Panel A and B shows characteristic hydroxyapatite deposition of the elastic lamina of coronary arteries and intimal proliferation in GACI patients with ABCC6 mutations or with ENPP1 mutations, respectively (von Kossa staining, 100x magnification). Panel C and D shows yellowish papules on the cervical region, a characteristic symptom in PXE, in patients with mutation in ABCC6 and in ENPP1, respectively. Panel E and F: X-ray image of the shoulder showing periarticular calcification in patients with mutation in ABCC6 and ENPP1 respectively. This figure was taken from Nitschke et al, 2012.

The genocopy, i.e. the overlapping phenotype observed between the two diseases suggest that these entities most probably reflect the two end of a clinical spectrum rather than two distinct pathologies and also indicates the existence of additional downstream mediators in this pathway leading to soft tissue mineralization.

II.3. Soft tissue calcification:

For the better understanding of calcification progresses underlining the pathology of PXE and GACI diseases I follow the advise of my supervisor (A.V.) and review soft tissue calcification progresses in great detail taking special attention to genes involved in the process.

Ectopic calcification is a progressive pathologic mechanism of mineralization affecting tissues throughout the body. Calcium and phosphorous salts, in most of the cases hydroxyapatite (HA) crystals [Ca10(PO4)6(OH)2], deposit within soft tissues or cartilages. Soft tissue calcification that affects arteries, the aorta, cardiac valves or the myocardium is a serious risk factor of cardiovascular diseases. There are three types of pathologic calcification: 1.) dystrophic soft-tissue calcification, which is the most common cause of soft-tissue calcification representing 95-98% of the cases; 2.) metastatic calcification; and 3.) calcinosis. Dystrophic calcification occurs within normal metabolic conditions in damaged or devitalized tissues; usually no associated metabolic disorder is identified. Metastatic calcification affects previously undamaged, normal tissues and it is a result of elevated calcium or other salt levels in the circulation. It is frequently associated to metabolic disorders; e.g. to hyperparathyroidism, to hypervitaminosis D or to chronic kidney disease. Calcinosis affects cutaneous, subcutaneous or deep connective tissue regions; usually no consistent metabolic disturbance can be observed or the ethiology is unknown. It might be associated to collagen-vascular diseases.

II.3.1. The two main types of arterial calcification:

Arterial calcification has progressive pathology that underlines many diseases. It is not restricted to blood vessels but also may affect the myocardium and the cardiac valves, where it predominantly occurs at sites of inflammation and mechanical stress. Calcium phosphate deposits may occur in different layers of blood vessels that is characteristic to specific pathologies. Intimal atherosclerosis is a plaque-like, discontinuous calcification, see Fig6.a, observed in atherosclerotic lesions [Hunt et al, 2002; Burke et al, 2000;

Giachelli et al, 2009]. Medial calcification, also termed Mönckeberg's calcification, is a uniform wall calcification of the lamina media, see fig 6.b. It is associated to age related arterial calcification, also observed in diabetes mellitus and in end stage renal disease (ESRD)/chronic kidney disease [Mönckeberg, 1903; Edmonds et al, 1982; Micheletti et al, 2008; Giachelli et al, 2009].

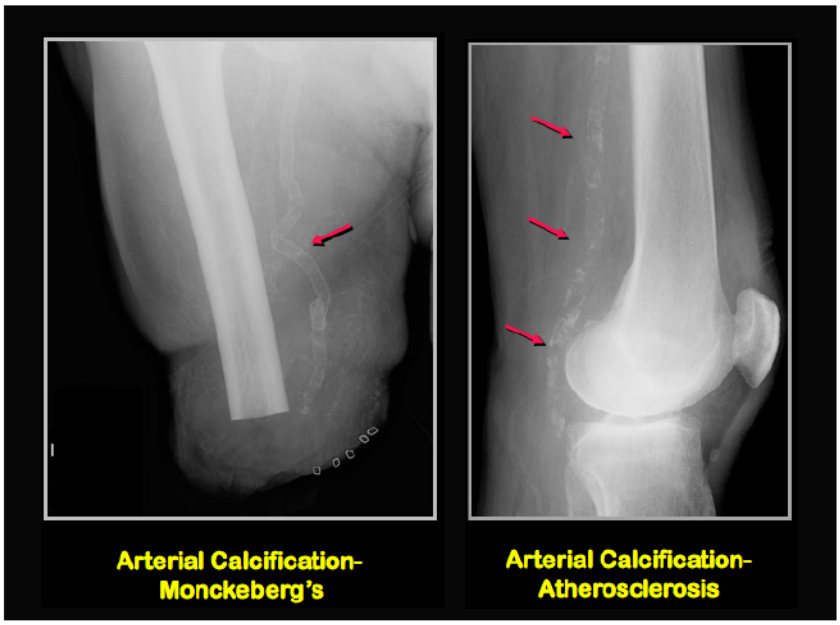

Fig.6: Radiological images of arterial calcification: Fig1a: X-ray image of Mönckeberg's medial calcification; Fig1b: X-ray image of intimal atherosclerotic plaques. Images were taken from Learning Radiology.com

The third type of vascular calcification is the calcific valvular stenosis that is thought to be an atherosclerotic process affecting aortic valves. The most prominent pathology in PXE and GACI diseases is the calcification of elastic fibers in the lamina media of arteries, corresponding the Mönckeberg's calcification.

Pathologic processes of medial calcification (Mönckeberg's calcification) are thought to be independent of atherosclerosis, and it was shown to resemble embryonic ossification [Sage et al, 2010] through the phenotypic plasticity of vascular smooth muscle cells.

Both intimal and medial vascular calcification contributes to the morbidity and mortality of cardiovascular diseases and thus is in the focus of intense scientific interest. Due to the increased stiffness and thus the decreased compliance of vessels, medial arterial calcification correlates with the increased risk of cardiovascular events and coronary artery disease [Giachelli et al, 2009]. The carrier status of the most frequent PXE-causing loss of

function ABCC6 mutation (c.3421C>T; p.R1141X) was shown to be associated with a higher risk for coronary artery disease (CAD) in two studies [Trip et al, 2002; Köblös et al, 2010], while a third revealed contradictory results suggesting no correlation [Hornstrup et al, 2011].

II.3.2. Basic regulators of the Ca and phosphate homeostasis, the pro-calcific / anti-calcific balance

In vertebrates extracellular body fluids are supersaturated with calcium and phosphate, thus calcium phosphate precipitates were previously thought be a result of passive unregulated process as the consequence of degenerative changes during aging. In the past decade complex regulatory mechanisms of vascular calcification were recognized. Vascular calcification is a culmination of several pathologic processes; multiple genes as well as environmental factors are known to contribute to the progress. Inflammatory mechanisms, alteration in metabolic processes, oxidative stress, hyperlipidemia and degeneration of the extracellular matrix components are known to promote calcification [Golub, 2011]. Control mechanism involves a delicate balance between pro-calcific and anti-calcific processes [Giachelli et al, 2009]. Anti-calcific factors include molecules like pyrophosphate (PPi) and several small molecular weight protein such as fetuin A, Matrix Gla protein (MGP) and further Vitamin K dependent proteins (described later, see chapter 5.), ordinarily restricted to biomineralization of skeletal bone [Sage eta l, 2010]. The most important pro-calcific factor is inorganic phosphate (Pi). The endocrine system mediated Ca homeostasis is a well-known process, it is mediated primarily by major calcium-regulating hormones and receptors: parathyroid hormone (PTH)/PTH receptor (PTHR) and 1,25-dihydroxyvitaminD/vitaminD receptor (VDR) and the Ca sensing receptor (CaR). The hormonal system regulating phosphate levels involves two main hormones: fibroblast growth factor 23 (FGF-23) and the FGF/Klotho receptor complex and it is also controlled by the calcium regulating PTH/PTHR system [Peacock, 2010]; however changes in serum phosphate level are well tolerated within a wide range. Marked fluctuation in the phosphate levels upon meals can be observed in the body; moreover children have much higher values than adults. In case of disrupted FGF-23R/Klotho hyperphosphataemia develops that leads to ectopic soft tissue calcification [Peacock, 2010]. Similar to *Enpp1-/-* mice (described later), disrupted bone development and marked ectopic calcification was observed in *Fgf23-/-* animals [Sitara et al, 2004; Stubbs et al, 2007; Mackenzie et al, 2012].

Renal excretion of both calcium and the phosphate is well regulated. Current theories describing basic regulatory processes in ectopic calcification are summarized on Fig 7.

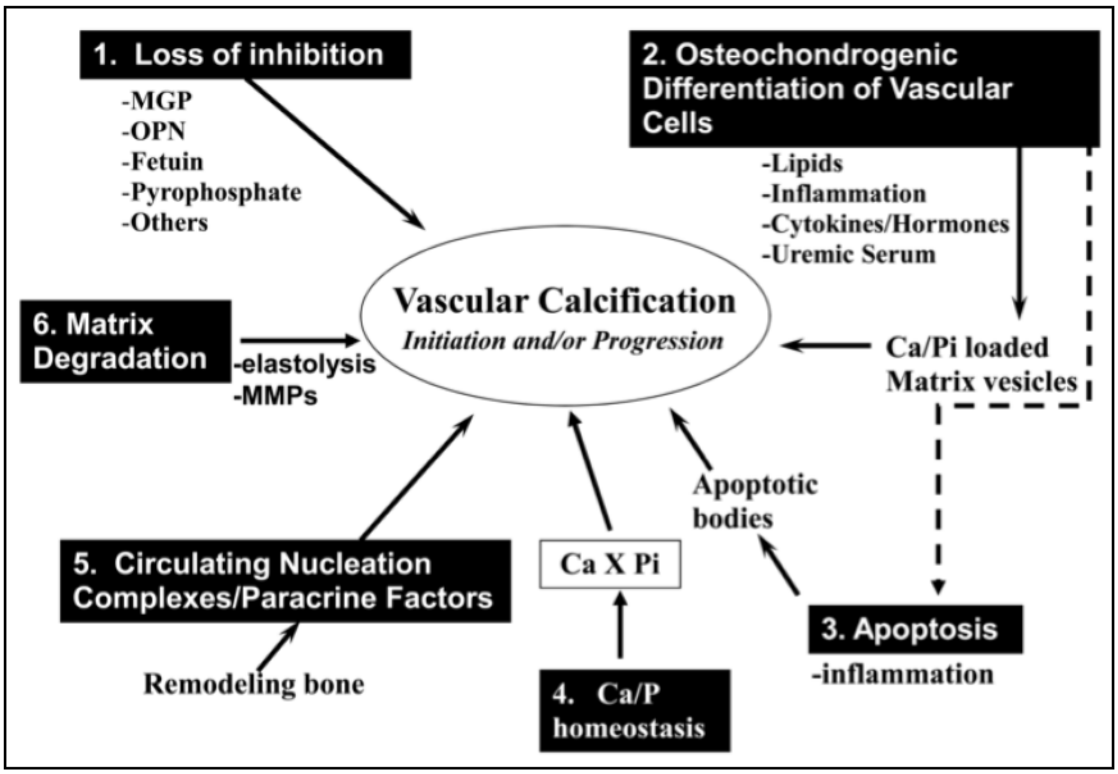

Fig.7: Theories on the regulation of vascular calcification, figure was taken from Giachelli et al, 2009.

II.3.3. Mendelian disorders highlighted crucial players of vascular calcification processes

Susceptibility to vascular calcification appears to be largely dependent upon genetic factors. It was concluded by Rutsch et al that approximately 50% of the variance in aortic calcification and in coronary calcification are related to genetic factors [Rutsch et al, 2011]. Vascular calcification is characteristic to several single gene Mendelian diseases but it also underlines a variety of complex metabolic disorders like hyperlipidemia, chronic kidney disease, diabetes, hyperpararthiroidism and osteoporosis. Single Mendelian disorders may highlight crucial steps of pathways regulating normal calcium and phosphate balance or mediating inhibitory mechanisms of ectopic calcification. Inbred mouse strains also show different occurrence of medial calcification in coronary arteries or atherosclerotic plaque formation if kept on atherogenic diet [Qiao et al, 1994,1995; Wang et al, 2007; Giachelli et al, 2009]. Table 2., adapted from Giacelli et al, lists the human

genetic syndromes and mouse mutations identified so far in association with vascular calcification. These genes may regulate calcification processes in blood vessels and other soft tissues.

The local ratio between PPi and Pi is mediated by proteins that ordinarily regulate bone mineralization: ectonucleotide pyrophosphatase/phosphodiesterases (NPPs), the tissue nonspecific alkaline phosphatase (TNAP), type III sodium-dependent Pi co-transporter (PiT-2), see Fig8., and the ankylosis protein (ANK). NPP1 hydrolyses ATP in order to generate either PPi and AMP or Pi and ADP. Pi promotes precipitation of HA crystals, while PPi inhibits HA formation, however it is also a precursor for Pi. PPi is a more potent inhibitor of the HA formation than the activator potential of Pi. The main role of TNAP is the breakdown of PPi; besides it also produces Pi. Both PPi and Pi inhibit TNAP activity and induce osteopontin (OPN) that inhibits mineral formation via limiting HA crystal growth. Transport of PPi and Pi through the cell membrane is mediated by ANK and PiT-2 proteins, respectively.

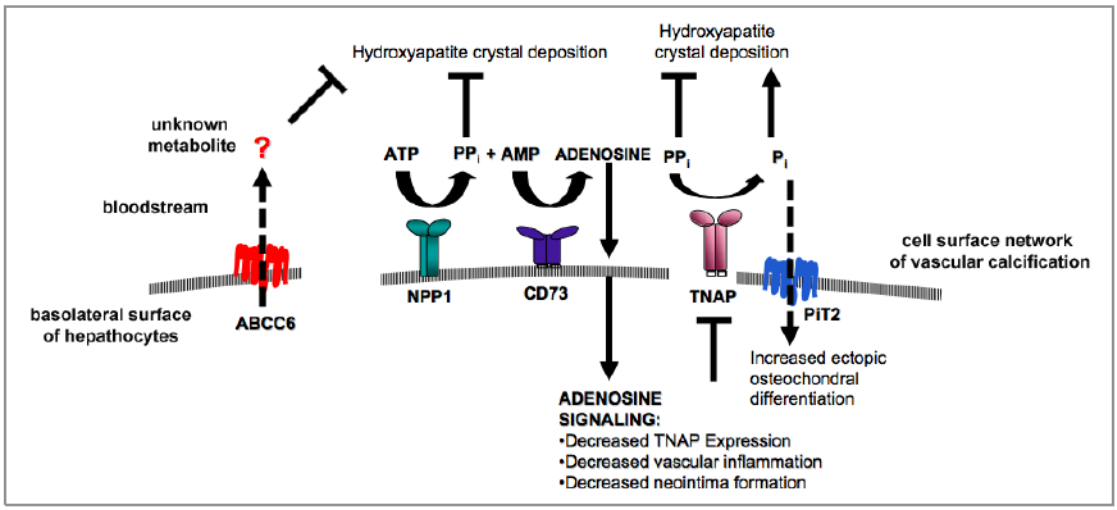

Fig.8: Theoretical network promoting and preventing vascular calcification in a balanced process as it is concluded on the basis of Mendelian disorders associated to soft tissue calcification. This figure is a modified version of a figure published in the paper Nitschke et al, 2012. Ectoenzyme NPP1 generates AMP and PPi from ATP that are hydrolized by CD73 and TNAP in order to generate adenosine and Pi. Pi is a component of hydroxyapatite depositions. PPi is an effective suppressor of hydroxyapatite deposition. Adenosine signaling suppresses TNAP expression and it inhibits vascular inflammation. The role of the basolateral hepatic transporter, ABCC6 and that of its substrate in the network remains unclear.

Biomineralization overall in the body is characteristic to tissues that express collagen I and alkaline phosphatase (ALP or TNAP). Type I collagen but not ALP is present in the normal artery wall [Murshed et al, 2005; Sage et al, 2010]. However in atherosclerotic plaques ALP was shown to co-localize with collagen I in calcific deposits [Sage et al, 2010]. A theoretical network promoting and preventing vascular calcification is summarized on Fig8 (see above).

Mutations in the *ENPP1* gene and in the encoded NPP1 protein are the genetic bases of generalized arterial calcification in infancy (GACI; OMIM: 208000) [Ruf et al, 2005; Rutsch et al, 2003], also described in the previous section. The NPP1 enzyme is expressed at the cell surface of many cells, including cartilage, heart, kidney, parathyroid and skeletal muscle, and it is also highly expressed in the vascular smooth muscle cells, osteoblasts and chondrocytes [Mackenzie et al, 2012]. The large catalytic extracellular domain of the enzyme can be cleaved and it may function as a secreted circulating protein. Infants with GACI disease have low serum level of PPi, they develop calcification of the lamina interna in large and medium sized arteries that cause stenosis. Vascular smooth muscle cell proliferation was also observed in the disease. Patients born with GACI usually do not survive beyond six months. Mouse models of the disease are the so-called „tiptoe walking mouse" (*ttw/ttw*) [Okawa et al, 1998], with homozygous stop codon mutation of the NPP1 protein and the transgenically engineered *Enpp1-/-* mice [Sali et al, 1999] that recapitulate calcification symptoms of the disease. Homozygous or heterozygous mutations of *ENPP1* in an ApoE background were observed to attenuate atherosclerosis but enhance medial calcification in mice [Nitschke et al, 2011]. GACI disease and animal models highlighted the important role of pyrophosphate in the pathologic background of vascular calcification.

It is important to mention that not only the down regulation of NPP1 but also the over-expression can induce mineralization as it was demonstrated on human valve interstitial cells [Mackanzie et al, 2012]. In a recent study Mackenzie et al demonstrated elevated levels of circulating fibroblast growth factor 23 (FGF-23) in *Enpp1-/-* mice [Mackenzie et al, 2012b]. This result was in harmony with human genetic studies reporting on hypophosphataemic rickets as a result of elevated FGF-23 levels in humans with *ENPP1* mutations. Other single gene mutations related to bone mineralization and thus phosphate homeostasis, were also shown to cause changes in FGF-23 gene transcription.

Previously mutations in the *ABCC6* gene were described as the genetic basis of pseudoxanthoma elasticum (PXE; OMIM: 264800), a recessive genetic disorder with characteristic calcification symptoms of the skin, lamina media of arteries and the Bruch's membrane within the eye, also described in the previous section. *ABCC6* -/- mice recapitulate features of pseudoxanthoma elasticum, they develop ectopic calcification of several tissues including the skin, arterial blood vessels, the retina, and the vibrissae [Klement et al, 2005; Gorgels et al, 2005]. It was also suggested by Jansen et al that *Abcc6-/-* mice may have reduced levels of plasma PPi [Jansen et al, 2013]. The *Abcc6* locus was also associated to the Dyscalc1 phenotype in mice [Meng et al, 2007] that is characteristic to dystrophic calcinosis at sites of inflammation and necrosis, suggesting a precursor role for medial disruption in calcification process [Wang et al, 2009].

Recently ABCC6 mutations, overlapping PXE-associated mutations, were shown to be also causative in the more severe GACI disease [Nitscke et al, 2012]. Interestingly Nitschke et al also observed four GACI patients carrying biallelic mutations of ENPP1 gene who developed typical signs of PXE later in their childhood [Nitscke et al, 2012]. The genocopy observed between the two diseases suggest that these entities most probably reflect the two end of a clinical spectrum rather than two distinct pathologies and also indicates the existence of additional downstream mediators in the pathway.

The cell surface protein CD73, encoded by *NT5E* gene, catalyzes the conversion of AMP to adenosine and inorganic phosphate (Pi). *NT5E* gene was shown to be associated to vascular and joint calcification in three families (CALJA; OMIM: 211800) [St. Hilaire et al, 2011; Nitschke et al, 2012]. The phenotype of patients with loss of function mutations of *NT5E* is significantly milder than those of with *ENPP1* mutations. Although the produced Pi is a procalcifying agent, adenosine is thought to be a potent suppressor of the gene expression of tissue non-specific alkaline phosphatase (TNAP) enzyme responsible for the reduction of PPi levels. As a result of the reduced extracellular adenosine levels and the enhanced activity of TNAP arterial calcification may develop. Based on the similarities of the degeneration of elastic fibres in PXE and CALJA diseases Markello et al suggested adenosine as a substrate for ABCC6 [Markello et al, 2011]. However *in vitro* measurements could not support this theory [Szabo et al, 2011].

It was also concluded that distinct arterial regions are more or less susceptible to different levels of pyrophosphate, inorganic phosphate or adenosine; moreover additional

protein factors might also contribute to the prevention of calcification of arteries or supplement loss of function mutations of *NT5E* gene [Hofmann B and McNelly, 2012].

The gene *SLC20A2* causative in Familial idiopathic basal ganglia calcification (IBGC, OMIM: 114100), is also involved in the phosphate/pyrophosphate homeostasis encoding a type III sodium-dependent phosphate transporter (PiT-2). IBGC is a rare disorder with characteristic calcification of brain areas and vasculature. *SLC20A2* is widely expressed but with marked high levels in the brain [Nitscke et al, 2012].

The four genes, *ENPP1, ABCC6, NT5E, and SLC20A2*, causative in the above mentioned four genetic disorders, are likely to be involved in physiologic processes related to ATP metabolism, inorganic pyrphosphate, adenosine and inorganic phosphate generation or transport [Nitscke et al, 2012].

Gene	**Mouse mutant phenotype**	**Human genetic mutation/phenotype**
Matrix Gla Protein	Arterial, valvae and cartilage calcification (Luo, 1997)	Keutel Syndrome/cartilage and soft tissue calcification (Hur, 2005)
Fetuin	Low serum HA inhibitory activity, enhanced susceptibility to vitamin D overload-induced vascular calcification (Schafer, 2003)	None reported
Osteopontin	Increased calcification of implanted bioprosthetic valve tissue (Steiz, 2002, Ohri, 2005), increased vascular calcification in OPN -/- XMPG-/- mice (Speer, 2002)	None reported
Osteoprotegerin	Vascular calcification and osteoporosis (Bucay, 1998)	None reported
FGF23	Hyperphophatemia, high serum Vitamin D, vascular calcification (Stubbs, 2007)	Familiar Tumoral Calcinosis/vascular calcification, hyperphosphatemia, high serum vitamin D (Benet Pages, 2005)
Klotho (b-glucuronidase)	Vascular calcification, rapid aging (Kuro-o, 1997)	Tumoral calcinosis, hyperphosphatemia (Ichikawa, 2007)
Nucleotide pyrophosphatase *Enpp1/PC-1/NPP1*	Tip toe walking mouse/ vascular and articular cartilage calcification (Okawa, 1998)	Infantile Arterial Calcification/low pyrophosphate, extensive vascular calcification, neonatal lethal (Rutsch, 2003)
Ank (pyrphosphate transporter)	Progressive Ankylosis, Articular cartilage calcification, soft tissue calcifcation (Harmey, 2004)	Calcium Pyrophosphate Deposition Disease/chondrocalcinosis, high pyrophosphate (Yaka, 2006)

Carbonic Anhydrase II	Small artery VC, osteoporosis, metabolic acidosis (Spicer, 1989)	Osteoporosis, metabolic acidosis, brain calcifications (Shah, 2004)
Smad6/Madh6	Endocardial cushion defect, valvular calcification (Galvin, 2000)	None reported
Desmin	Neonatal cardyomiopathy with calcification (Mavroidid, 2002)	None reported
UDP N–acetyl-a-D-galatosamine (GalNT3)	None reported	Familiar Tumoral Calcinosis/hyperphophatemia, vascular calcification, elevated serum vitamin D (Ichikawa, 2005)
Fibrillin1	Marfan-like syndrome, elastocalcinosis and aneurysm	Marfan Syndrome
Fibulin4	Valve calcification and stenosis, aortic dilatation	Cutis laxa, Aortic aneurysm, perinatal lethal (Dasouki, 2007)
ABCC6 transporter	Extensive soft tissue and vibrissae calcification (Klement, 2005)	Pseudoxanthoma elasticum/calcification of skin, connective tissue and vasculature (Le Saux, 2000)
BMP and Receptor	BMP4 overexpressing transgenic, fibrodysplasia ossificans-like phenotype (Kan, 2004)	ACVR1 BMP receptor, activating mutations, Fibrodysplasia Ossificans Progressiva/muscle/soft tissue calcification (Shore, 2006)
WRN RecQ helicase	Accelerated mortality in p53 null background (Lombard, 2000)	Werner's syndrome/soft tissue calcification (Uhrhammer, 2006)
Lamin A (*LMNA*)	Cardiac and skeletal myopathy; progressive loss of vascular SMC and calcification (Varga, 2006)	Hutchinson-Gilford Progeria/ calcification associated with atherosclerosis (Eriksson, 2003)
Glucocerebrosidase (D409H)	Lysosomal storage disorder, valve calcification not found (Xu, 2003)	Gaucher Disease/lysosomal storage disease, valvular and aortic arch calcification (McMahon, 2001)
Transcription Intermediatery Factor (*TIF1 alpha*)	Ectopic calcification including arterioles and medium sized arteries (Ignat, 2008)	None reported
Calcium Sensing Receptor (*CaSR*)	Gprc2aNuf mouse, activating mutation, Ectopic calcification including arterial calcification and cataracts (Hough, 2004)	Activating mutations, autosomal dominant hypocalcemia, hupercalciuria, and Bartter-like syndrome (Pollak, 1994)
CD73/NT5E	Vascular proinflammatory phenotype (Blume et al, 2012)	Calcification of joints and arteries, CALJA (St. Hilaire et al, 2011)
SLC20A2	disturbed renal reabsorption of Pi (Myakala, 2014)	Basal ganglia calcification, idiopathic 1 (Wang et al, 2012)

Table 2. Genes associated with vascular calcification in Men and Mice. Loss of function mutations are shown unless stated otherwise. This table is a modified version of the table published in Giachelli et al, 2009.

II.3.4. Vascular smooth muscle cells of the arterial lamina media may develop osteochondrogenic phenotype

Under normal conditions deposition of calcium phosphate within soft tissues is a thermodynamically unfavorable mechanism, pyrophosphate (PPi) prevents the formation of precipitates. [Hofmann Bowman and McNelly, 2012] Hydroxyapatite crystals deposit during bone formation where they grow from extruded matrix vesicles (20-200nM particles) that bud from the surface of osteoblast cells and not only serves as nidus for bone formation but also provides a milieu optimal for HA crystal formation [Anderson et al, 2003; Hofmann Bowman and McNelly, 2012]. Matrix vesicles may offer a microenvironment with high calcium and phosphate levels while PPi levels are low. They are also significantly enriched in tissue nonspecific alkaline phosphatase [Harmey et al, 2004; Sage et al, 2010; Golub, 2011], the NPP1 enzyme and type III sodium-phosphate co-transporters [Golub, 2011] that accumulate phosphate within the cells/vesicles. Type III sodium-phosphate co-transporters are widely expressed, e.g. in the kidney, liver, lung, heart, brain osteoblast, chondrocyte and smooth muscle cells [Giachelli et al, 2009].

Molecular events of vascular calcification were shown to resemble the enchondral ossification of bone. Different vascular cell types were demonstrated to undergo osteoblastic calcification, to up regulate TNAP and to produce matrix vesicle-like particles. In *Enpp1-/-* background phenotypic transition of VSMCs, i.e. the up-regulation of chondrogenic differentiation associated molecules was observed during aortic calcification. VSMCs were also shown to exude matrix vesicles *in vitro* that promoted calcification. TNAP inhibitors were demonstrated to block calcification mediated by VSMCs. These vesicles share some of their protein content with bone derived matrix vesicles. [Kapustin et al, 2011; Hofmann Bowman and McNelly, 2012; Golub, 2011]. Inflammation, hyperlipidemia, elevated phosphate levels and mechanical stress are factors that are likely to play a role in stimulating a signaling cascade that turns vascular cells to undergo osteoblastic differentiation. Pro-calcification molecules, like BMPs, may play a role in the reprogramming [Jono et al, 2000; Giachelli et al, 2009; Sage et al, 2010; Golub, 2011]. Cytokines may increase the expression of sodium-dependent phosphate transporters in SMCs and thus lead to calcification processes even at normal levels of phosphate within tissues. Elevated phosphate level was shown to be a strong inducer of vascular calcification *in vivo* on rat aortas and in SMC cultures [Giachelli et al, 2009]. Mineral

deposits in cardiac valves co-localize with mechanical stress [Sage et al, 2010; Schoen et al, 2009]. Indicators of greater stiffness, like fibronectin induced osteochondrogenic differentiation, while indicators of distensibility like laminin were shown to promote smooth muscle/adipogenic differentiation [Sage et al, 2010; Yip et al, 2009]. Cross-linking of matrix proteins increases matrix stiffness, thus cross-linking agents may also contribute to the development of vascular calcification. Pathologic processes leading to arterial remodeling are illustrated on Fig.9.

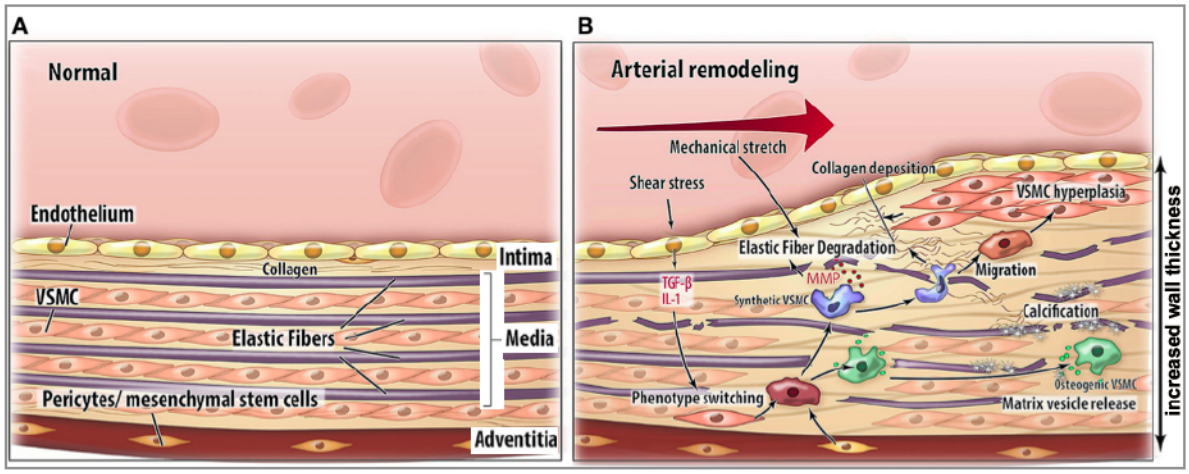

Fig.9: Pathophysiological mechanisms of arterial remodeling. A: schematic view of a normal arterial wall. B: Arterial remodeling. Characteristic features are thickening, elastic fiber degeneration, extracellular matrix calcification and collagen deposition. Stress factors, matrix metalloproteinases (MMPs) as well as the phenotypic transition of vascular smooth muscle cells (VSMCs) are involved in the process. This image was taken from van Varik et al, 2012.

II.3.5. NPP1 as signal regulator in calcification processes

NPP1 has been demonstrated to act not only as an ectopic enzyme but also as a regulator in wide range of signalling pathways. It also may modulate arterial calcification through RAGE receptor that is a receptor of advanced glycation end products (AGE) that are result of nonenzymatic glycation and oxidation of proteins. These are ligands that were shown to accumulate in disease conditions related to extracellular matrix degeneration processes. RAGE is widely expressed; it has been detected on endothelial cells, macrophages, dendritic cells, smooth muscle cells and other vascular cell types [Hofmann and McNelly, 2012]. Through RAGE signaling transcription factors like NFkB, ERK1/2, MAPK and oxidative stress signaling are activated resulting in aortic matrix remodelling

[Hofmann et al, 1999]. If *Enpp1* null mice was generated on a RAGE deficient background less arterial calcification developed compared to *Enpp1* -/- *Rage* +/+. However these mice did not show the rescue of the skeletal phenotype of *Enpp1*-/- mice, indicating that the Npp1–RAGE signaling might be specific to vascular smooth muscle cells [Mackenzie et al, 2012]. These experiments further proved that oxidative stress and chronic inflammation are involved in phosphate-mediated soft tissue calcification processes [Cecil and Terkeltaub, 2011]. A reduced production of the RAGE suppressor molecule was detected in Enpp1-/- aortic ring cultures. Treatment of this culture with the suppressor inhibited calcification and chondrogenic differentiation [Cecil and Terkeltaub, 2011; Mackenzie et al, 2012].

Glycation end products (AGE) that act through the RAGE receceptors and mediate oxidative stress linked degenerative processes are also characteristic degenerative processes in the macula, the retinal region severely affected in most of the PXE patients.

II.3.6. Calcification linked pathologic processes in the Bruch's membrane of the eye

The most severe consequence of PXE is the calcification of the elastic laminae of the Bruch's membrane in the macula that leads to the fragmentation of elastic fibers and triggers choroidal neovascularization. As a consequence many patients suffer from severe visual impairment. Choroidal neovascularization in PXE is thought to resemble the neovascular form ('wet form') of age-related AMD that may result in a sudden vision loss.

The Bruch's membrane (BM) is an acellular layer located between the retinal pigment epithelium (RPE) and the fenestrated choroidal capillaries of the eye. It is an elastin- and collagen-rich extracellular matrix. Both the choroid and RPE cells are capable of synthesizing the major components of the BM [Booij et al, 2010]. Sivaprasad et al suggested a common origin of BM and the vascular intima, the tissue affected by the symptoms of PXE other the eye [Sivaprasad et al, 2005]. The structure of the BM is different in the macular area, a 6-mm-diameter region that serves for fine acuity vision, compared to the retinal periphery. Examining human donor eyes of all ages, Chong et al found that the elastic layer of the BM was three to six times thinner in the macula and it was more porous than in the peripheral region [Chong et al, 2005]. Microarray studies of human donor eyes age of 17-36 years by other also found regional differences in the gene expression of 33 structural BM proteins in RPE cells [van Soest et al, 2007]. BM acts as a semi permeable filter, it regulates the exchange of biomolecules, nutrients, oxygen, fluids

and metabolic waste products between the retina and the circulation. The molecular, structural and thus the functional properties, e.g. the permeability, of the Bruch's membrane are dependent on age and genetic background and are also affected by environmental factors. With age calcification of elastic fibers, cross-linkage of collagen fibers and the turnover rate of glycosaminoglycans increases; advanced glycation end products (AGEs), wastes and lipids accumulate, see Fig.10. Advanced glycation end products are glycosylated or oxidized fats and proteins. AGEs frequently accumulate on structural proteins, like collagen molecules, where they inhibit function and are associated with age-related damage. High concentration of AGE compounds in the serum or within tissues activate AGE receptors (RAGE), present on multiple cell in the body, which may initiate inflammatory mechanisms [Booij et al, 2010]. These are sources of prolonged oxidative stress and lead to the controlled activation of the complement system in the healthy balanced system to clear up debris in BM. Lymphocytes are also able to infiltrate the retina even in case of an intact blood-retina-barrier (BRB), if lymphocytes are activated they may initiate the transient breakdown of the BRB and initiate further inflammatory processes. If balanced homeostasis can no longer be maintained age-related macular degeneration (AMD) develops. Similar pathology can be observed in genetically determined ophthalmic disorders, like PXE. Features of aging and diseases may overlap, distinction between normal aging and pathology in AMD is not clear [Booij et al, 2010].

The negatively charged proteoglycans (heparan, chondroitin and dermatan sulphate), abundant in the BM, have a property to bind water and positively charged cations, like sodium, potassium and calcium; and also form a barrier for negatively charged molecules. Heparan sulphate also has anti-inflammatory properties [Meri and Pangburn, 1994; Booij et al, 2010]. Changes in the characteristics of proteoglycans in the BM with age may modify the natural binding properties of inhibitory complement molecules at the surface. Van der Schaft et al detected calcium deposition in 59% of the analysed samples from 33 years old healthy individuals, and the extent of calcification was positively correlated with age [van der Schaft et al, 1992]. Extensive calcification makes elastic fibers brittle thus it leads to breaks and consequent neovascularization [Booij et al, 2010]. Leukocytes, lymphocytes, macrophages and the endothelial cell also may contribute to the breakdown of collagen and facilitate neovascularization [Penfold et al, 1987]. Maccari et al measured altered polysaccharide content of the urine in PXE patients as compared to healthy

individuals; chondroitin sulfate was significantly decreased, while heparan sulfate was significantly increased [Maccari et al, 2003]. These data are in agreement with the findings of Logas et al, who described abnormal glucose amino glycans (GAGs) in the urine and in the lesional skin of PXE patients [Longas et al, 1986].

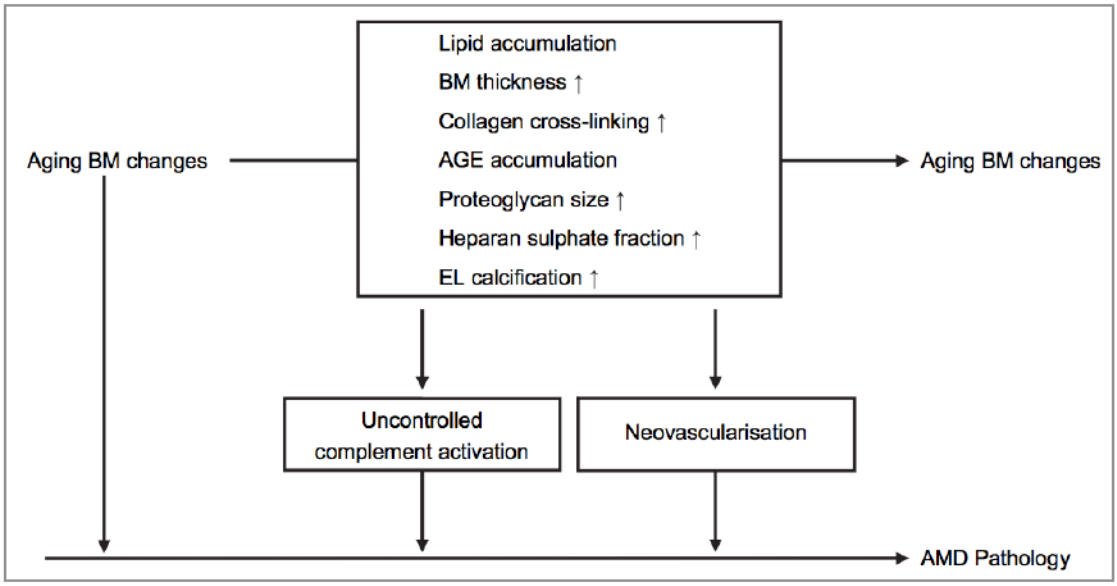

Fig.10: Age related changes in the Bruch membrane (indicated in the upper frame) that might lead to the development of AMD. Figure is taken from Booij et al, 2010.

II.3.7. Preventive and therapeutic efforts targeting soft tissue calcification in PXE and GACI diseases

The physiological substrate of ABCC6 protein still remains unknown, consequently no specific and effective clinical treatment that could reverse or prevent systematic mineralization process in PXE is currently available. However the principal role of NPP1 in soft tissue calcification is clear, there is no effective therapy for classical GACI disease either. Preventive approaches targeting hyperphosphatemia were adapted from other mineralization disorders, like chronic kidney disease. Bisphosphonates, synthetic analogues of inorganic pyrophosphate, such as etidronate, were already started to use in case of GACI patients. The treatment successfully reduced calcifiacation deposits in infants with GACI disease, however it cannot avert early death in all cases and spontaneous regression of ectopic calcification was also observed [Cheng et al, 2005; Mackenzie et al, 2012]. Based on succesful experiments with *Abcc6-/-* mice

overexpressing transgenic α-fetuin, Uitto suggested approches targeting the delivery of anti-calcification proteins into the circulation of PXE patients [Jiang et al, 2010; Uitto, 2014]]. Preventive and therapeutic efforts have been adapted in PXE for the eye manifestations from clinical trials in AMD focusing on the inhibition of STAT3/VEGF induced neovascularization. Based on a genetic association study of VEGF polymorphisms in PXE patients Zarbrock et al suggested the potential involvement of VEGF in the pathomechanism of PXE-related choroidal neovascularization [Zarbock et al, 2009]. Choroidal neovascuarization was successfully treated in PXE patients when bevacizumab VEGF inhibitor was administered intravitreally. [Bhatnagar et al, 2007]. Best results were achieved when treatment started in the earliest stages of the disease [Finger et al, 2011], visual acuity could be maintained or even improved [Verbraak, 2010]. However, currently intravitreal anti-VEGF treatment is the best choice for patients with choroidal neovascularization, it is worth to mention that anti-VEGF therapy may increase the risk of cardiovascular and/or thromboembolic events.

Numerous studies declared the mineral composition of the diet being a modifier of the severity of the symptoms. High intake of calcium and phosphate might lead to the more severe manifestation of PXE [Renie et al, 1984]. In *Abcc6-/-* mouse model dietary magnesium was shown to influence ectopic mineralization. 5 fold increase in the magnesium content of the diet completely abolished mineralization, while reduction of magnesium to 20% of the normal value resulted in accelerated calcium deposition [LaRusso et al, 2009; Jiang et al, 2012]. Clinical trials of the high magnesium diet in PXE are in progress [Uitto, 2014].

In vivo preclinical research in mice targeting folding deficient disease associated variants of the ABCC6 protein found in PXE and GACI patients are discussed in the last chapter of the results part of my thesis. Those experiments could lead to a personal based allele specific therapy in PXE and GACI diseases. Since the correctional therapy targets ABCC6 protein throughout the body, this therapy might result in the systematic prevention or reverse of ectopic mineralization.

II.4. Short overview of our knowledge about ABCC6 protein as a transporter

In 2002, Iliás et al and Belinsky et al successfully expressed the human ABCC6 protein in Sf9 insect cells, and in Chinese hamster ovary cells (CHO), respectively. They performed *in vitro* transport measurements on membrane vesicles derived from the latter

cells and revealed ABCC6 as an organic anion transporter capable for the transport of glutathione S-conjugates, such as leukotriene C4 (LTC4), S-(2,4 dinitrophenyl) glutathione (DNP-SG), and N-ethylmaleimid S-glutathione (NEM-GS) but not for glucuronate conjugates [Iliás et al, 2002; Belinsky et al, 2002]. Organic anions (probenecid, benzbromarone, indomethacin), known to interfere with the transport of gluthatione-conjugates in case of ABCC1 and ABCC2 proteins, were demonstrated to inhibit the NEM-GS transport in a specific manner, characteristic to ABCC6 [Iliás et al, 2002]. The estimated Km and Vmax values of the LTC4 transport were 600nM and 50pmol/mg membrane protein/min, respectively; while the apparent Km and Vmax values of the NEM-GS transport were 282±54 μM and 106±8 pmol/mg membrane protein /min, respectively [Iliás et al, 2002]. Concentration dependent LTC4 uptake of ABCC6 is illustrated on Fig.11.

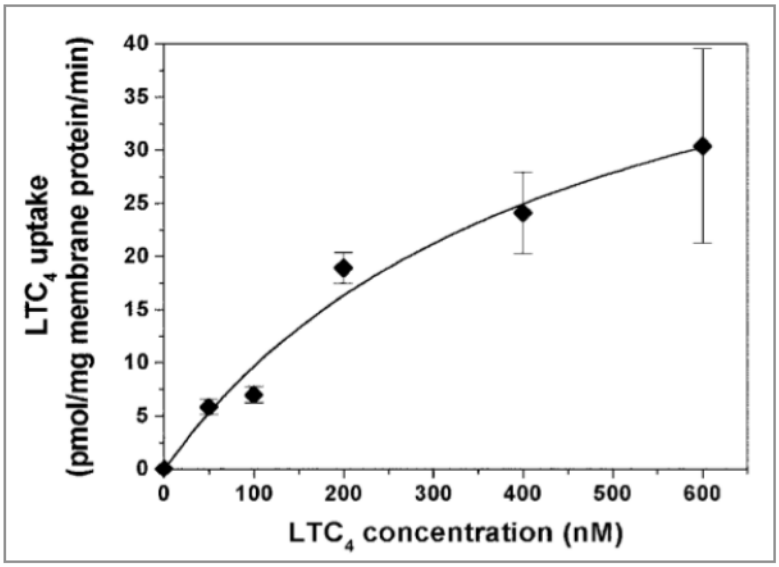

Fig.11: Concentration dependence of LTC4 uptake by membrane vesicles prepared from Sf9 insect cells expressing human ABCC6 transporter. The rate of transport was measured after 30 s incubation time, at 37°C. These are similar conditions as used in transport measurements targeting the VitaminK tranport capacity of ABCC6 transporter analyzed in this thesis. Figure was taken from [Iliás et al, 2002].

Only a marginal transport for 17-β-estradiol-17-β-D-glucuronide (E217βG), a prototypical glucuronide conjugate, could be detected [Iliás et al, 2002]. Similar uptake results were shown by Belinsky et al. They obsereved approximately 0.5 pmol/mg membrane protein/min uptake at 20nM LTC4 concentration for ABCC6 protein. For DNP-SG they

detected approximately 3.5 pmol/mg membrane protein/min transport at 1μM DNP-SG concentration. No transport for the E217βG (1μM) compound could be detected [Belinsky et al, 2002].

Previous to human ABCC6 the rat Abcc6 transporter was shown to transport a small anionic cyclic peptide, BQ-123 endothelin receptor antagonist, known to attenutate endothelin-1 induced vasoconstriction. The appearent Km value of the rat Abcc6 for BQ-123 was 17μM that is 7 fold less than the Km of the Abcc2 transporter, indicating a much higher affinity of rat Abcc6 to this compound as compared to rat Abcc2 [Madon et al, 1999]. Human ABCC6 was also demonstrated to be able to transport the BQ-123 compound by both groups, however the transport activity was much lower than that of the rat transporter, maximum transport was estimated to be 6.5pmol/mg membrane protein/min, while Km could not be determined [Iliás et al, 2002]. Belinsky et al measured a transport value approximately 1-1.5 pmol/mg membrane protein/min at 1μM of BQ-123 [Belinsky et al, 2002].

Attila Iliás also demonstrated that missense mutations can result in the loss of transport activity and the ATP-binding/hydrolyzing ability of the protein. Amino acid substitutions affecting conserved motifs of the catalytic domains (V1298F, G1302R, G1321S) were investigated. Mutant proteins were analyzed in vesicular transport experiments as well as in nucleotide-binding and nucleotide-trapping assays [Iliás et al, 2002].

II.5. The molecular background of PXE-like syndrome; vitamin K forms as potential substrate candidates for ABCC6 transporter

II.5.1. The molecular background of PXE-like syndrome

In 2007, a disease with similar cutaneous symptoms to PXE but with additional vitamin K-dependent coagulation factor deficiency has been described by Vanakker et al [Vanakker et al, 2007]. They could not identify any causal mutations in the *ABCC6* gene, responsible for pseudoxanthoma elasticum, or in the *VKORC1* (vitamin K 2,3 epoxide reductase) gene, see Fig.12, frequently affected in patients with vitamin K-dependent coagulation factor deficiency. The molecular analysis revealed mutations in the second gene involved in clotting disorders, the *GGCX* (γ-glutamyl carboxylase) gene in six out of seven patients [Vanakker et al, 2007]. The GGCX enzyme is responsible for the γ-carboxylation of gla-proteins. Gla-proteins are Ca-chelating proteins. They harbour

glutamic acid reach "Gla-domains"; γ-carboxylation of these residues is required for function [Theuwissen et al, 2012a].

Extracellular fluids in vertebrates are supersaturated with calcium and phosphate. Potent inhibitors of calcium phosphate formation are essential for survival. Low molecular weight inhibitors are pyrophosphate and citrate, but the most potent inhibitors are small and medium-sized proteins. Gla-proteins are also termed as vitamin K dependent proteins, since Vitamin K compounds are essential co-factors for the γ-glutamyl carboxylase (GGCX) enzyme, see Fig.12.

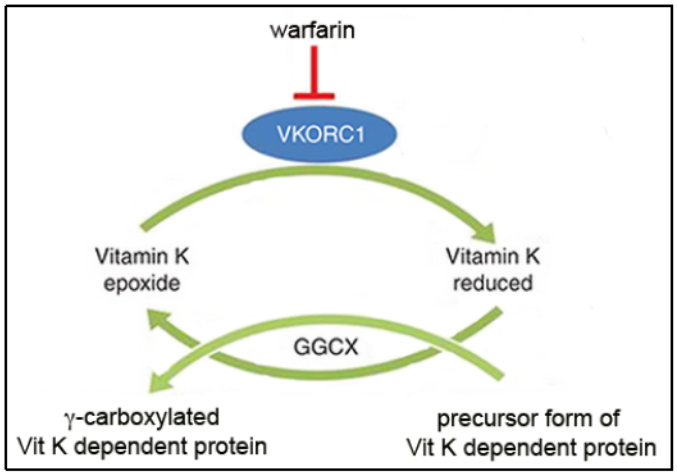

Fig.12: Reduced Vitamin K is essential cofactor for γ-glutamyl carboxylase (GGCX), the enzyme resposible for the activation of Gla-proteins. Warfarin inhibits vitamin K epoxide reductase complex subunit 1 (VKORC1), responsible for the regeneration of reduced vitamin K. Figure is a modified version of that published in [Turner et al, 2013].

The most important inhibitor of arterial calcification is the Matrix-gla protein (MGP). MGP is synthesized by numerous cell types, including vascular smooth muscle cells [Gheduzzi et al, 2007]. MGP prevents the precipitation of hydroxyapatite crystals at the elastic lamellae [Theuwissen et al, 2012a]. MGP-knockout mice die from massive aortic and coronary calcification symptoms short after birth [Luo et al, 1997]. Vitamin K dependent proteins also play essential role in the maintance of osteo-articular system, teeth, and in the regulation of normal cell growth. Un-carboxylated forms of vitamin K dependent proteins (17 identified so far), like MGP, are biologically inactive; elevated levels in the blood are signs of relative or absolute vitamin K deficiency [Vanakker et al, 2007]. MGP levels in the serum and in dermal fibroblast of PXE patients were analyzed in

multiple studies; significantly reduced amounts of MGP was detected, especially that of the fully maturated γ–carboxylated form (Glu-MGP), as compared to control samples [Gheduzzi et al, 2007; Vanakker et al, 2010]. In PXE skin biopsies the un-carboxylated form (Gla-MGP) was localized in the peripheral regions of the calcium phosphate precipitates [Gheduzzi et al, 2007]. These findings were in harmony with studies on the *Abcc6*-/- knockout mice; the calcified vibrissae of the animals contained exclusively un-carboxylated MGP [Jiang et al, 2007; Li et al, 2007]. Previous observations on human aortas with atherosclerosis revealed similar results; Glu-MGP was co-localized with the calcification while Gla-MGP could be detected only around the elastic fibers [Schurgers et al, 2005]. Based on the above findings and on the molecular background of PXE-like disease a research hypothesis has been concluded that the slowly progressing calcification symptomes in PXE might be due to a peripheral vitamin K deficiency [Borst et al, 2008]. Animal skin graft transplantation studies [Jiang et al, 2009] and the parabiotic mouse model of *Abcc6-/- and Abcc6* +/+ mice proved PXE as being a metabolic disease [Jiang Q et al, 2009], suggesting an essential role of the liver in PXE.

Dietary vitamin K is transported in the blood via chylomicron particles. The most prominent proportion of these is efficiently taken up and utilized by the liver. Tissues at the periphery are less well provided with vitamin K [Theuwissen et al, 2012]. Significant amount of Vitamin K forms are temporarily stored in the liver and are subsequently secreted into the bile and most probably into the blood. Vitamin K metabolites, especially the metabolised/conjugated forms, are good substrate candidates for the ABCC6 basolateral hepatic transporter.

As also expressed in the proximal tubules of the kidney, it was speculated that ABCC6 might have a function in preserving vitamin K compounds in the circulation [Borst et al, 2008]. Due to this hypothesis, the loss of function mutations of ABCC6 may lead to peripheral vitamin K deficiency and to the progressive calcification symptoms of the elastic tissues in PXE. Vitamin K supply is supposed to be normal in the liver of PXE patients, the organ in which coagulation factors are synthesized, thus they do not develop blood coagulation problems.

II.5.2. The route and the role of different vitamin K forms in the body

The core structure of the vitamin K compounds is 2-methyl-1,4-naphthoquinone; also called menadione or VitK3, see Fig.13. This form does not occur in the nature but possesses biological activity in vertebrates. Geranylgeranyl side chain can be added at the 3' position forming MK-4, a form of vitamin K2, see Fig.13, thus menadion may be regarded as a provitamin [Shearer et al, 1996]. Naturally occuring vitamin K forms all have the menadion core structure and differ from each other in the side chain added at the 3' position. Plants synthesize only one major form that has the same phytyl side chain as chlorophyll, and it is termed phylloquinone, vitamin K1. Bacteria synthesize a group of compounds called menaquinones, vitamin K2, with side chains of repeated prenyl units. The nomenclature is based on the number of prenyl units: MK-n, see Fig.13. Partially saturated forms are also synthesized by some bacteria, they are abbreviated to: MK-n(H_2), MK-n(H_4) and so forth [Shearer et al, 1996].

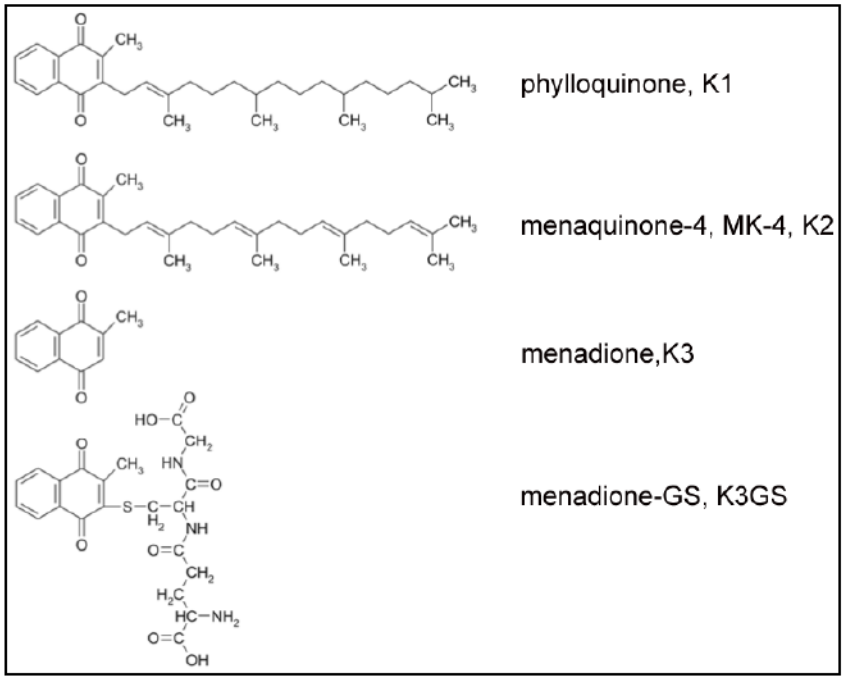

Fig.13: Basic structures of vitamin K forms and that of the glutathionyl-menadione (VK3GS) conjugate.

Phylloquinone is most abundant in dark-green vegetables and in vegetable oils. Food sources of bacterially synthesized menaquinones with possible nutritional significance are the livers of ruminant animals (MK-6 - MK-13) [Shearer et al, 1996]; eggs and fish (MK-4). Other sources of the long-chain menaquinones are the fermented foods (bacterial),

typically cheeses and curds (MK-8, MK-9); and natto (MK-7). Estimates suggest that approximately 90% of the total vitamin K uptake comes from phylloquinone, 7.5% from MK-5-9 and 2.5% from MK-4 [Schurgers et al, 2000]. Very long-chain menaquinones (MK-10-13) are known to be synthesized by the anaerobic genus *Bacteroides* that are major inhabitants of the intestine [Shearer et al, 1996]. Gut menaquinones most probably contribute to the total vitamin K pool as minor source [Suttie, 1995].

Dietary vitamin K forms are absorbed in the proximal intestine by bile salt-mediated pathway that operates for dietary lipids [Shearer et al, 1974]. After intestinal absorbtion phylloquinone is incorporated into chylomicrons (CM) and chylomicron remnants (CR), a form of triglyceride-rich lipoproteins (TRL); through lymphatic lacteals it enters the blood. The most prominent proportion of CRs is efficiently taken up by the liver, the organ where the coagulation factors, required for immediate survival, are synthesized. Tissues at the periphery are less well provided with vitamin K, the major source of these are vitamin K2 forms. Vitamin K insufficiency occurs sooner and in a more pronounced form in extrahepatic tissues [Theuwissen et al, 2012].

Vitamin K compounds are utilized in the activation of gla-proteins, they serve as cofactor for the γ-glutamyl carboxylase (GGCX) enzyme. The reduced form of the vitamin Ks (KH2) is the active co-factor. During this redox reaction KH2 is converted to vitamin K-epoxide (KO). Dithiol dependent reductases catalyze the recycling of the epoxide form. Coumarin derivatives, such as warfarin, inhibit the activity of the reductases [Fasco et al, 1982], see Fig.12.

While the lack of vitamin K, especially the phylloquinone (K1), causes severe blood coagulation deficiency, according to a current hypothesis, vitamin K2 deficiency might be responsible for the so called “calcium paradox”, a pathologic phenomenon, that is the lack of calcium in the bone and its storage in the vessel walls [Flore et al, 2013]. This may result in cardiovascular accidents and bone fractures. Vitamin K2 is essential in the maintance of the normal structure of arterial wall; it plays crucial role in the inhibition of vascular calcification. Calcific atherosclerosis is a significant determinant in cardiovascular diseases. Prolonged anticoagulant treatment with dicoumarols (vitamin K antagonists) is also associated with the increased risk of vascular and cartilaginous calcifications, and non-traumatic bone fractures [Flore et al, 2013]. A population-based study in the Netherlands suggested protective role of dietary menaquinone intake against

coronary heart disesease (CHD) in older men and women, while phylloquinone intake did not show any association with CHD, mortality or aortic calcification [Geleijnse et al, 2004]. In a study of Spronk et al, MK-4 was shown to prevent warfarin-induced vascular calcification if rats were kept on a diet containing warfarin and K1 paralelly that induced vascular calcification within 4 weeks. Even high doses of K1 could not prevent vascular calcification [Spronk et al, 2003]. Extrahepatic tissues, such as the aorta, were observed to utilize MK-4 more efficient than K1 [Spronk et al, 2003].

The low levels of circulating MGP and its impaired carboxylation within tissues is associated with the development of cardiovascular disease [Schurgers et al, 2005].

A population genetic study from our laboratory revealed that the carrier status of the most frequent PXE-causing loss of function ABCC6 mutation (c.3421C>T; p.R1141X) is associated with higher risk for coronary artery disease (CAD) [Köblös et al, 2010] that may suggest a role of ABCC6 in vitamin K dependent calcification processes.

It is interesting to note that in contrast to the sources of phylloquinone, the menaquinone sources, like eggs, red meat, and high-fat cheeses are not related to healthy lifestyle diet, which is often suggested to follow for persons with high risk of vascular calcification diseases. The 10-40% of circulating major Gla-proteins (MGP, osteocalcin) was shown to be un-carboxialted in healthy adult population, which is an indirect sign that western diet contains insufficient amount of vitamin K [Theuwissen et al, 2012].

Phylloquinone stores of the body are labile and relatively low compared to vitamin A, D and E. The catabolism is extensive; 60-70% of a single administered phylloquinone dose, independent upon the range (45µg-1mg), is ultimately excreted as catabolic products. 20% of the injected labelled phylloquinone was excreted in the urine and 40-50% was excreted in the faeces via the bile within 3 days. In the bile it was detected as polar metabolites, they did not find evidence of re-absorbtion [Shearer et al, 1974]. The so far identified urinary metabolites of phylloquinone are glucuronide conjugates [Shearer and Berkhan, 1973; Shaerer and Newman, 2008]. Thijssen et al found that after oral doses of either phylloquinone or menaquinone forms menadione-conjugates appear in the urine within one hour, indicating a phylloquinone to menaquinone conversion [Thijssen et al, 2006].

Many extrahepatic tissues contain high levels of MK-4, a form that is not abundant in dietary sources. Since the 1960s it was a question whether the source of it is the gut flora or local conversion through free menadion in tissues. The final proof came from the

laboratory of Thijssen and Suttie who proved that germ-free rats are able to convert phylloquinone to MK-4 equally as good as control animals [Ronden et al, 1998; Davidson et al, 1998 Shaerer and Newman, 2008]. In other studies MK-4 was found to be synthesized de novo in cell lines HEK-293 (human embryonic kidney), and primary cultures of mouse cerebral hemispheres if menadione was administered into the culture media [Davidson et al, 1998 Okano et al, 2008 Shaerer and Newman, 2008]. Using deuterium labelled phylloquinone, both on the ring and on the side-chain, Okano et al proved that MK-4 was synthesized from phylloquinone through complete side-chain removal [Okano et al., 2008], indicating the involvement of free menadione as intermedier in the process. Whether the source of menadione is the liver, it still remains unknown.

It was demonstrated in *in vitro* measurements by Shukla et al that menadione is a substrate of ABCG2 [Shukla et al, 2007]. Since ABCG2 is an apical hepatic transporter this may have physiologic importance. As being a hydrophobic toxic intermedier it is highly probable that menadione is also converted to phase II conjugation product(s) and it is subsequently excreted via apical or basal transporters from hepatocytes.

It has been demonstrated by Di Monte et al that rat hepatocytes are able to form menadione-glutathione conjugate (VK3GS) and secret it into the medium *in vitro* [Di Monte et al, 1984]. It was not a minor reaction since 15% of the cellular GSH was converted into VK3GS. After menadion perfusion of rat liver mainly glutahionyl-menadione but menadione-glucuronide as well were shown to be excreted into the bile [Losioto et al, 1968; Akerboom et al, 1988]. It is still a question, whether VK3GS is also transported into the blood. However VK3GS is likely to be a good substrate of a basolateral organic anion transporter, like ABCC6, already proved to transport glutathione conjugates *in vitro* [Iliás et al, 2002, Belinsky et al, 2002]. VK3GS retained in the circulation might serve as source of MK-4 synthesis at the periphery. If this hypothesis were true, it would elucidate the role of ABCC6 in soft tissue calcification and thus in the pathologic background of PXE and GACI diseases. However we cannot rule out the contribution of other hepatic transporters, like ABCs, to the VK3GS export either. Potential ABC type transporter candidates, already shown to be transport glutathione conjugates, are ABCC2 [Oude et al, 1989; Paulusma et al, 1999] and ABCG2 apical transporters facing the bile canaliclule; and ABCC1 [Jedlitschky et al, 1994; Leier et al, 1994; Loe et al, 1996; Deeley et al, 2006], ABCC3 at the basolateral surface of

hepatocytes, however ABCC3 is rather a poor transporter of gluthatione conjugates [Zeng et al, 2000; Zelcer et al, 2001].

II.6. Mutational hot spots in DNA might be causative in genetic diseases

During my PhD period I also contributed to the establishment of the ABCC6 mutational database (http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6), and investigated the contribution of mutational hot spots, especially those of the 5'CpG dinucleotides, to the large amount of sequence variations of the *ABCC6* gene. According to this I give an overview here about the role of the 5'CpG dinucleotides frequently found in eukaryotic sequences.

Inherited mutations significantly contribute to chronic diseases. Mutations are either transmitted from carrier or from affected parents or may arise de novo in each generation. Although new mutations are relatively rare they are the sources of genetic variability. Source and dynamics of substitutional mutations are of great interest, since it may help in understanding the origin of many genetic disorders, particularly why some disease-causing mutations can be seen more often than expected [Arrnheim and Calabrese, 2010].

Mutational hot spots/functional elements: the role of CpGs in eukaryotic genome sequences

In vertebrate genome sequences cytosine residues located 5' to a guanosine, i.e. in 5'CpG dinucleotides, are frequently methylated by a methyltransferase enzyme. 5-methyl cytosines (5mC) constitutes approximately 0.7-3% of the bases of vertebrate cells and occurs almost exclusively within 5'CpG dinucleotides [Ehrlich and Wang, 1981]. 5mCs spontaneously get deaminated to thymine residues [Wang et al., 1982 Holliday et al., 1993]. Reaction with oxidative radicals or chemical agents can also lead to deamination. There is a repair mechanism, which specifically recognizes G-T mismatch sites and replaces T with C. However this repair is not efficient enough. Moreover G-T mismatches (result of deaminated 5mC) are excised less efficiently than G-U mismatches (result of deaminated C) from the DNA [Ehrlich et al., 1990; Holliday et al., 1993], thus the higher spontaneous mutational frequency at 5-methyl cytosines than at cytosines is likely due to the combination of these two mechanisms. Hence 5mC>T transitions occur about ten times more frequently than other transitions and such mutations are frequently observed in heritable diseases [Holliday et al, 1993]. In a study of Cooper and Youssoufian, single

nucleotide variations within coding regions of genes associated to heritable diseases were collected. 35% of mutations were found to be located to 5'CpG sites. More than 90% of these mutations were C>T or G>A. They estimated that the CpG dinucleotide is up to 42 times more mutable than other sequences [Cooper and Youssoufian 1988]. According to these 5'mCpG were declared as mutational hot spots [Bird et al., 1980a]. Most likely for this reason 5'CpG dinucleotides are significantly underrepresented in vertebrate genome sequences; in about 99% of the genomes occurring some of a quarter of the frequency expected presupposing random distribution of the bases [Sved and Bird, 1990].

Methylcytosine was first identified in DNA by Wyatt over 60 years ago. He analysed DNA samples of various animal and plant origin as well as DNA from viruses, bacteria and yeast. He found that all animal sources contain methyl cytosine, in an amount characteristic to the species. He could not isolate methyl cytosine from microbial sources. Based on the amount in which methylcytosin occurred in the analysed samples he denoted it as essential constituent of certain DNAs and no accident of enzyme action [Wyatt, 1951]. Wondering on the relevance of his observations he was the first who speculated on the functional role of these unstable nucleotides in the genome sequences of higher organisms. Later investigations further proved that vertebrate organisms have markedly reduced amount of CpGs compared to the estimated frequency of genome-wide nucleotide composition [Bird, 1986]; while Drosohila, C. Elegans and most procaryotes have relatively normal occurence of CGs. This phenomenon might be explained by the lack of the standard methylase in the latter organisms, although some other non vertebrates, lacking the methylase, like Neurospora and yeast also have reduced values of CGs [Burge et al., 1992]. Why did not have eukaryotes evolved an efficient mechanism to counteract the mutagenic activity of the methylase? Might the function of DNA methylation is to increase the mutational rate or it might have other function(s) [Bird, 1980a]? Methylcytosines increase the mutation rate, although this mechanism is shortsighted, as all the cytosines in CG dinucleotides would dissapear, unless a selection also works to preserve CpGs [Bird, 1980a]. By analysing the sequences of the human α–globin gene and its pseudogene Sven and Bird investigated and proved that the 20-25% frequency of CpGs represents equilibrium between the rate of newly generalized CpGs and the accelerated loss by methylation induced deamination [Sved and Bird 1990] supporting the existence of a mechanism that preserves CpGs.

Detailed studies highlighted regulatory roles of CpGs in gene expression mechanisms in eukaryotes. Cytosine DNA methylation is an epigenetic mechanism, has important role in gene silencing or activation, thus it is essential for normal development [Bird, 2002; Reik, 2007; Branciamore et al., 2010]. During development cells start in a pluripotent state and through differentiation states they progressively develop a narrower potential. Genes that are required later in development are repressed by histone marks. These confer short-term, flexible epigenetic silencing. DNA methylation confers long-term epigenetic silencing; it can be reprogrammed by demethylation, which might involve DNA repair. Little is known about how epigenetic marks influence lineage commitment in development [Reik, 2007].

There are genomic regions, termed CpG islands (CGIs) that escape CpGs suppression and comprises CpG clusters longer than 200bp, have above 50% G+C content, and a CpG frequency almost as high as it is expected if presupposing random distribution [Bird et al., 1986; Burge et al., 1992; Schorderet et al., 1992; Vinson et al, 2012]. These regions are predominantly unmethylated, thus genetically stable, and represent approximately 1% of the genome; and harbour 5% of all CpGs [Cooper et al., 1983; Vinson et al, 2012]. CG rich regions usually represent CGI promoters associated with ubiquitously expressed housekeeping genes [Vinson, et al 2012; Bird, 2002; Saxonov et al., 2006] that activate essential cellular functions. Basically CGI promoters are thought to be protected against methylation by the binding of certain protein factors such as Sp1 [Medvedeva et al., 2010], but most probably other mechanisms also might have a role [Saxonov et al., 2006]. The remaining 95% of the CGs are disposed throughout the 99% of the genome and are typically methylated, thus genetically unstable. Interestingly, these unstable areas may also serve as functional promoters, nearly half of all promoters, and activate tissue-specific gene expression thus play essential role in embryonic development, gene imprinting and X chromosome inactivation [Bird, 2002;Vinson, et al 2012; Branciamore et al., 2010]. Methylation of the CGI promoters (originally unmethylated) results in the suppression of gene activity via the reduced availability for binding factors [Iguchi-Ariga et al., 1989; Tate et al., 1993; Rozenberg et al., 2008; Vinson et al, 2012]. On the other hand methylation of the low CG content promoters is required for activation; it facilitates binding of C/EBP family of transcription factors [Vinson et al, 2012, Rishi et al., 2010].

CpGs are also disposed throughout the coding region and are highly methylated, thus genetically unstable. Exons are usually more GC-rich than introns [Aissani et al., 1991;

Saxonov et al., 2006; Medvedeva et al., 2010]. It is especially true for polypeptide sequences with specific amino acid composition, e.g. with many arginine codons that harbour CpG dinucleotides. CpGs are directly embedded within eight triplets, see Fig.14, highlighted in red. Four of these code for amino acids: Ser, Pro, Thr, Ala. In half of these cases C>T or G>A replacement would result in amino acid alteration. The remaining four codones code for Arg, in which all mutational events would result in amino acid replacement. CpGs may also be formed by two neighbour codones and thus serve as potential mutagenic hot spots. These CpGs contribute to the large number of single nuleotide mutations observed in genetic diseases [Holliday et al, 1993].

1	2								3
	T		C		A		G		
T	TTT	Phe	TCT	Ser	TAT	Tyr	TGT	Cys	T
	TTC	Phe	TCC	Ser	TAC	Tyr	TGC	Cys	C
	TTA	Leu	TCA	Ser	TAA	stop	TGA	stop	A
	TTG	Leu	TCG	Ser	TAG	stop	TGG	Trp	G
C	CTT	Leu	CCT	Pro	CAT	His	CGT	Arg	T
	CTC	Leu	CCC	Pro	CAC	His	CGC	Arg	C
	CTA	Leu	CCA	Pro	CAA	Gln	CGA	Arg	A
	CTG	Leu	CCG	Pro	CAG	Gln	CGG	Arg	G
A	ATT	Ile	ACT	Thr	AAT	Asn	AGT	Ser	T
	ATC	Ile	ACC	Thr	AAC	Asn	AGC	Ser	C
	ATA	Ile	ACA	Thr	AAA	Asn	AGA	Arg	A
	ATG	Met	ACG	Thr	AAG	Lys	AGG	Arg	G
G	GTT	Val	GCT	Ala	GAT	Lys	GGT	Gly	T
	GTC	Val	GCC	Ala	GAC	Asp	GGC	Gly	C
	GTA	Val	GCA	Ala	GAA	Glu	GGA	Gly	A
	GTG	Val	GCG	Ala	GAG	Glu	GGG	Gly	G

Fig.14: Table of the genetic code. 5'CpG dinucleotides embedded within eight triplets are highlighted in red. This figure is a modified version originally published in Branciamore et al, 2010.

During my PhD period I have also contributed to the generation of ABCC6 homology models and investigated the distribution of disease-associated mutants on functional surfaces of the protein predicted by the models. According to this I give here a short summary about the basis of homology modeling.

II.7. The basis of homology modeling; the predictivity of homology modeling in human diseases

The ultimate goal of homology modeling is to predict the atomic-resolution structure of a protein without experimental crystallisation data using the coordinates of (a) closely

related homolog(s). During evolution, protein structure is more stable and changes much slower than the related amino acid sequence, i.e. distantly related sequences may fold into similar structures. This relationship was first described by Chothia and Lesk [Cothia et al., 1986]; it serves as the basis of homology model building.

Generally homology modeling arranges the backbone of the target sequence according to that of the template. Every time model building begins with aligning our sequence of interest to the template(s). The quality of the sequence alignment has crucial importance. The so-called "safe zone" of modeling, based on the relationship between the length of the sequences and the amino acid identity of them, is illustrated on figure15. This figure was taken from the tutorial: "Homology Modeling" in Structural Bioinformatics [Krieger et al, 2003]

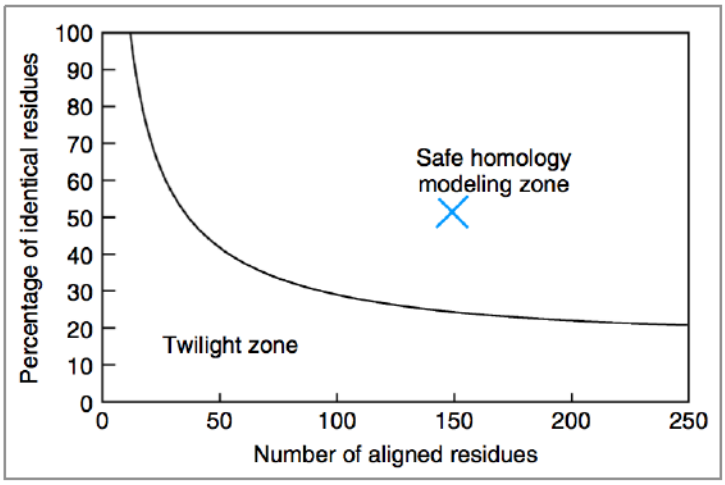

Fig.15: The two zones of homology modeling. Two proteins, those of the aligned sequences based upon the length and percentage of their sequence identity fall into the region marked "safe homology modeling zone", practically fold into the same structure. An example of two sequences with the length of 150 residues and 50% identity is indicated with blue cross on the figure (figure was taken from the tutorial: "Homology Modeling", Krieger et al., Structural Bioinformatics, 2003).

Misplaced sequences with gaps representing insertions or deletions will cause residues to be misplaced in space. There are programs available online that make alignments automatically. However, supervision by someone with specialized knowledge may improve the quality of the alignment, and hence the quality of the model.

During evolutionary time amino acid residues of the transmembrane helices in transmembrane proteins are more frequently replaced (by other 'helix compatible' amino

acids) than the residues of cytoplasmic domains, thus the sequence identity of the transmembrane regions are usually much lower than that of the whole molecule of related proteins. In case of large transmembrane proteins with multiple membrane spanning domains, like the ABC proteins, amino acid identity of the target and that of the template sequence is often less than 30%. However, because of the length of these sequences the overall reliability of modeling still may fall into the 'safe' zone. According to this, the predictivity of our model will be rather low in case of transmembrane regions but might be high in the region of cytosolic domains, especially those of the conserved regions.

In general, homology models are predictive at the level of alpha carbon positions in the folding. They can guide structure and function hypotheses, e.g. we can identify potential structural elements of the protein or important residues, which are predicted to localize in close proximity to regions of interest. Homology models are unlikely to be useful in predicting details of side-chain positions, or potential interactions between side-chains of residues unless the amino acid identity of the target and the template is very high.

In case of proteins related to genetic diseases homology modeling may serve as tool in visualizing disease-causing mutations in the structure. We may highlight mutations that are localized to structural or functional domains of the protein; and thus we may obtain basic information that can help to elucidate structure and function evidences of the protein. On the other hand, disease associated mutations may drive our attention to previously unidentified functional elements of the protein. Based on homology modelling, it is very difficult, and in most of the cases we can say it is very unlikely to predict the functional or structural consequences of certain amino acid replacements described in the population, i.e. that sequence alterations found overall the protein are pathogenic or not.

II.8. The tissue distribution profile and the subcellular localization of ABCC6 protein

However Beck et al in 2003 detected a low widespread expression of ABCC6 protein and the mRNA in the human body with the highest level in the liver [Beck et al, 2005] others since than showed ABCC6 protein to be unambiguously associated with the basolateral membrane compartment of hepatocytes in mice, rat and human samples [Madon et al, 2000; Beck et al 2003; Gorgels et al, 2005; Le Saux et al, 2011] and to a much less extent in kidney proximal tubular cells and in MDCKII kidney cell line [Sinko et al, 2003; Beck et al, 2005; Gorgels et al, 2005].

It was demonstrated by different research groups that hepatocyte nuclear factor 4 (HNF4), a master regulator of metabolic genes in the liver, both in human [de Boussac et al, 2010] and in mouse [Douet et al, 2006] has crucial regulatory role in *ABCC6* and *Abcc6* gene expression. It binds to an evolutionary conserved site [de Boussac et al, 2010;Ratajewski et al, 2012]. ABCC6 is expressed only in tissues where HNF4 is present, indicating that this transcription factor regulates the tissue-specific expression of this gene [Arányi et al, 2013].

In a recent study Martin et al challenged this well defined localization paradigm, suggesting that human ABCC6 protein is localized to the mitochondria associated membrane (MAM) and not to the plamamembrane [Martin et al, 2012]. They performed subcellular fractionation followed by immunoblot and cell surface biotin-labeling of primary hepatocytes. To reveal the exact subcellular localization of ABCC6 in its natural tissue environment our research group together with Olivier Le Saux's laboratory performed parallel immunohistochemical experiments with several monoclonal antibodies on frozen sections of mouse and human liver samples. Immunohistochemical staining of frozen human liver samples labelled for ABCC6; for cadherine, a plamammebrane marker; and for ABCB11 apical transporter are shown on Fig.16. Human ABCC6 and mouse Abcc6 proteins were exclusively localized in the plasmamembrnane, while no co-localization with mitochondrial marker (cytochrome C oxidase) was detected [Pomozi et al, 2013 and 2014]. Labeling of Abcc6 protein in cultured primary hepatocytes of mice revealed similar data [Pomozi et al, 2013]. The latter authors suggested that fractionation experiments performed in the study by Martin et al, might led to inaccurate conclusion concerning the intracellular localization of the membrane-embedded ABCC6 protein; and that the lack of biotin labeling might be due to the few potential surface biotinylation targets, only two, potentially reactive lysine residues available in extracellular loops [Pomozi et al, 2013].

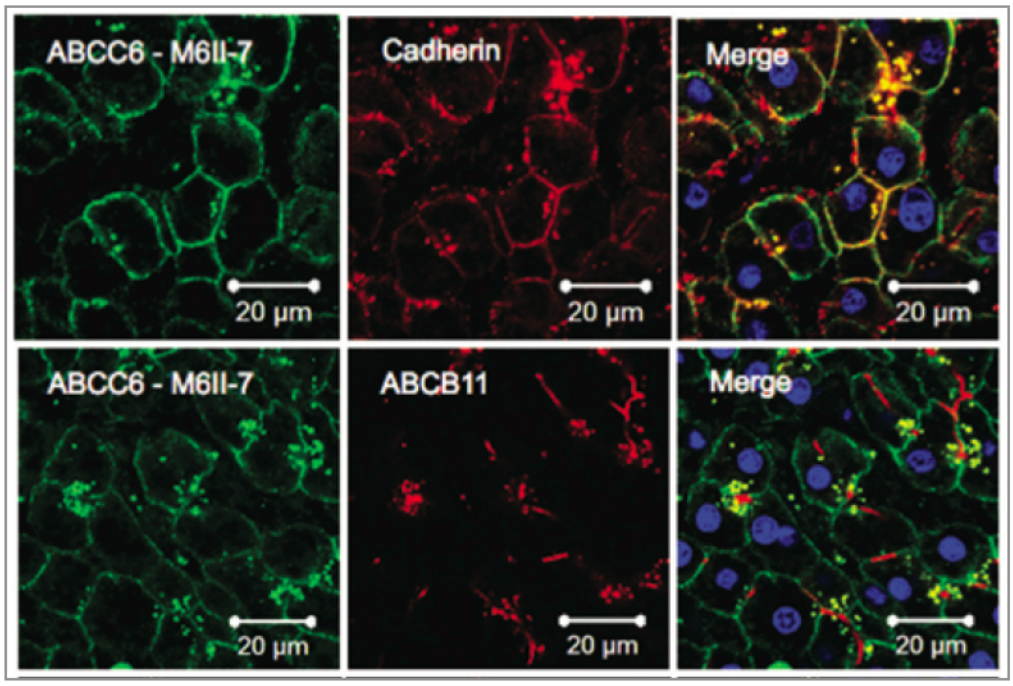

Fig.16: Immunofluorescence staining of frozen human liver sections, labelled with M6II-7 monoclonal antibody (green) and the plamamembrane marker cadherin (red) in the upper row; M6II-7 monoclonal antibody (green) and the anti-ABCB11 monoclonal antibody detecting ABCB11 apical transporter (red) on lower panels. Strong punctuate staining are nonspecific signals as evidenced by the negative control staining without primary antibodies, see Pomozi et al 2013.

Mechanism of chemical chaperones, correctors of folding deficiency; efforts toward allele specific therapy in PXE

In case of large membrane proteins, like ABCC6, mutations are very likely to disrupt protein stability/folding thus result in the miss-localization or altered half-life of the protein. According to these, protein cannot be maintained in efficient amount within its physiological target site in the cells. This explains one possibility how missense mutations may result in a recessive, loss of function phenotype as it is observed in heritable diseases associated to ABC transporters, e.g. cystic fibrosis (ABCC7), progressive familial intrahepatic cholestasis (ABCB11) and pseudoxanthoma elasticum (ABCC6).

Chemical chaperon molecules were previously demonstrated to efficiently correct unstable or miss-processed mutant proteins *in vitro* [Rubenstein et al, 2000; Zeitlin et al, 2002; Sorrenson et al, 2002;Cheong et al, 2006, Tveten et al, 2007, Hayashi and Sugiyama, 2007 and 2009; Lam et al, 2007; Powell et al, 2011]. Chemical chaperon molecules do not interact with proteins directly, thus they do not interfere with function [Ulloa-Aguirre and

Conn, 2011]. In our experiments published in our very recent paper [Pomozi et al, 2014], we focused on a chemical chaperon molecule, sodium 4-phenylbutirate (4-PBA) to investigate the rescue effect both *in vitro* and *in vivo* in mice. This compound is an FDA (US Food and Drug Administration) approved drug that might be a major advantage toward future clinical trials and allel specific therapy in PXE.

In the last two years two papers have reported on the first succesfull clinical use of this compound in two children with progressive familal intrahepatic cholestasis of infancy in Japan and in France [Gonzales et al, 2012; Naoi et al, 2014].

III. AIMS

Recently two human genetic diseases characterized by ectopic calcification symptoms are related to mutations in the *ABCC6* gene; pseudoxanthoma elasticum (PXE, OMIM 26480) and generalized arterial calcification of infancy (GACI, OMIM: 614473). The ABCC6 plasmamembrane transporter is predominantly expressed in hepathocytes and to a less amount in the kidneys. ABCC6 is thought to transport (an) unidentified substarte(s) involved in anti-calcification processes toward the circulation to peripheral tissues.

Molecular mechanisms underlining the background of the "PXE-like syndrome", a heritable diseases with similar peripheral calcification pathology related to mutations within the *GGCX* gene, advocated Vitamin K metabolites as substrate candidates of ABCC6 protein. In one part of my PhD period I focused on the functional properties of the transporter and investigated its potential involvement in the Vitamin K transport.

The other part of my work aims to analyze disease-associated mutations of the *ABCC6* gene and those of the transporter.

My specific aims were as follows:

1. To synthesize and to purify the radioactively labeled and the unlabeled GSH conjugated form of the VitK3 metabolite (VK3GS).

   To test ABCC6 transporter and additional hepatic ABC transporters, ABCC1, ABCC2, ABCC3, and ABCG2, for their ability to transport the VK3GS conjugate in *in vitro* vesicular transport measurements. I aimed to determine whether ABCC6 or any of the above hepatic ABC proteins is a transporter of the VK3GS conjugate.

2. Since there were already hundreds of disease-causing mutations described in the literature affecting the *ABCC6* gene thus far we decided to establish a validated database of the sequence variations.

3. In order to recall structure and function data embedded amongst the numerous disease-associated mutations we aimed to generate three dimensional homology models of the ABCC6 protein.

4. In order to analyze the consequences of disease-associated mutations of the ABCC6 transporter *in vitro* I have selected missense mutations that are frequent in PXE

and affect conserved functional or structural surfaces of the protein. I aimed to investigate their functional properties *in vitro* in vesicular transport measurements and to investigate their subcellular localization *in vitro* in MDCKII mammalian cells. As part of a collaborative work our specific aim was to identify transport competent but mislocalized mutants. These may serve as potential candidates in pharmacological rescue experiments targeting stability/folding correction of disease associated missense mutants both *in vitro* and *in vivo*.

IV. MATERIALS AND METHODS

MATERIALS

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. Restriction endonucleases and T4 ligase were obtained from New England Biolabs and Fermentas.

Vectors:

pAcUw21L Baculovirus vector (Pharmingen vector, modified by [Szakács et al., 2001])

SpSldS retroviral vector [Becker et al, 1998; Újhelly et al, 2003]

pLIVE (Mirus Bio, Madison, WI)

pEGFPN1 vector (Clontech)

pBluescriptSKII+ vector (Stratagene)

Primers used in PCR reactions were purchased from Metabion International.

Mutagenesis primer sequences:

delABCC6F: 5'-ggactcagatctgccaccatgggcggcagtggcatgaaggc-3'

delABCC6R: 5'-gccttcatgccactgccgcccatggtggcagatctgagtcc-3'

S1121WF: 5'-ccagctactggtctgtctgc-3'

S1121WR: 5'-gcagacagaccagtagctgg-3'

R1141XF: 5'-ggcattctgaacccaggcccc-3'

R1141XR: 5'-cctgggttcagaatgcccggacc-3'

T1301IF: 5'-gttggcaggatcggggcagg-3'

T1301IR: 5'-cctgccccgatcctgccaac-3'

Q1347HF: 5'-catcccccatgaccccatcc-3'

Q1347HR: 5'-ggatggggtcatgggggatg-3'

R1459CF: 5'-cattgcccactgcctgcgctc-3'

R1459CR: 5'-gagcgcaggcagtgggcaatg-3'

Primary antibodies:

Anti-human ABCC6 M6II-7 rat monoclonal (generous gifts from George Scheffer, University Medical Center Amsterdam)

Anti-human ABCC6 HB6 rabbit polyclonal (custom made peptide synthesis, Sigma)

Anti-human NaK-ATPase chicken polyclonal (Santa Cruz Biotechnology)

Secondary antibodies:

Anti-rat Alexa Fluor 488 and anti-chicken Alexa Fluor 594 (Invitrogen)

HRP-conjugated anti-rabbit and anti-rat antibodies (Jackson Immuno Research)

Radioactive reagents:

[^{3}H]LeukotrieneC_4 ([^{3}H]LTC_4) (130Ci/mmol) (Perkin Elmer),

[^{3}H]Estradiol-17b-D-glucuronide ([^{3}H]E_2-17G) (48.1Ci/mmol) (Perkin Elmer),

[^{3}H]methotrexate ([^{3}H]MTX) (24.0 Ci/mmol) (Moravek Radiochemicals, Brea, California, USA),

[^{3}H]GSH (36.6 Ci/mmol; Perkin Elmer)

Materials for transport measurements

Nitrocellulose membrane filters (HAWP02500) (Millipore),

Scintillation fluid (Opti-fluor) (PerkinElmer)

METHODS

Data processing of ABCC6 database

Published sequence variants of the *ABCC6* gene were collected from papers available in the PubMed database. Data were collected into Excel tables and Word files. The online database is powered by an LOVD platform; it was created in a collaborative work with PXE International and it operates on the server of the NCBI.

Protein sequence alignments

Protein sequence alignments of ABCC6, Sav1866, HlyB–ABC, CFTR–ABC and P-gp proteins were generated by ClustalW2 program [Larkin et al, 2007] using the default settings. For the closed conformation (outward facing) model building, using Sav1866, HlyB-ABC, CFTR-ABC as templates, the alignments showed the following amino acid identity data: the aligned sequences of ABCC6 TMD1–NBD1 and Sav1866 shares 23% identity, while the HlyB–ABC and CFTR–ABC has 31% and 35% identical amino acid positions with the N-ABC domain of ABCC6, respectively. The ABCC6 TMD2–NBD2 and Sav1866 is identical in the 23% of positions, the NBD2 of ABCC6 and the HlyB–ABC and CFTR–ABC shares 32% and 20% identity, respectively. In case of the open conformation (inward facing) model, using mouse P-gp as template, amino acid identity was as follows: the aligned sequences of ABCC6 TMD1–NBD1 and P-gp TMD1-NBD1

shares 20% identity, while in case of the ABCC6 TMD2–NBD2 and P-gp TMD2-NBD2 24% of the positions are identical. These data for the NBDS were: 28% for ABCC6 NBD1 and P-gp NBD1, and 34% for ABCC6 NBD2 and P-gp NBD2.

Model building

Homology model building was performed by using the Sav1866 bacterial ABC transporter coordinates (PDB Accession No: 2ONJ 3.4Å) as well as the coordinates of the HlyB ABC–ABC dimer with ATP-Mg and those of the N-ABC CFTR homodimer (PDB Accession No: 1XEF 2.5Å, 2PZE 1.7Å, respectively) in case of the closed conformation (outward facing) model and the coordinates of the mouse P-gp (PDB Accession No: 3G5U 3.8Å) in case of the open conformation model. The closed conformation model covers amino acid residues of human ABCC6 300–856 representing the second transmembrane and the first ABC domains (TMD1–ABC1) as well as 943–1503 representing the third transmembrane and the second ABC domains (TMD2–ABC2). The open conformation model covers amino acid residues of human ABCC6 37-856 representing the second transmembrane and the first ABC domains (TMD1–ABC1) as well as 907-1503 representing the third transmembrane and the second ABC domains (TMD2–ABC2). Two hundred structures were generated using the Modeller 9.3 software package [Sali and Blundell, 1993]. The one with the lowest objective function of the 200 generated models were used in the present study. Figures were made with PyMOL software. Fisher exact test was used for statistical calculations.

DNA constructs of ABCC6 variants

p.S1121W, p.T1301I, p.Q1347H and p.R1459C mutants were genereted by overlap extension mutagensis PCR method in wt cDNA containing pAcUw21L baculovirus transfer vector. Mutagenesis primers are listed in the materials section. Mutagenesis cassettes were subcloned using SfiI/NotI restriction endonucleases back to the original vector and validated by sequencing. The pAcUw21L vector constructs were used to express ABCC6 mutants in Sf9 (Spodoptera frugiperda) insect cells. The cDNA cassettes harbouring the above PXE-associated missense mutations were subcloned with BglII/NotI restriction enzymes into SpSldS retroviral vector [Becker et al, 1998; Újhelly et al, 2003] and used for *in vitro* expression in MDCKII cells. The same enzymes were used to subclone the above mutants into pLIVE vector. These constructs were used for *in vivo*

experiments in mice. Mutation containing cassettes were subcloned from pLIVE into pBluescript SKII+ plasmid as BglII/XhoI fragments to BamHI/XhoI compatible sites.

R1141X and Δ(2-275)ABCC6 mutants were generated by QuikChange Site Directed Mutagenesis method, first described by Stratagene, in pLIVE and pEGFPN1 vectors, respectively. Mutagenesis primers are listed in the materials section. Mutations were validated by sequencing, mutagenesis cassettes were subcloned back into the original cDNA containing vector and subsequentially into pLIVE and pBluescript SKII+ vectors as SfiI/XhoI fragments. These constructs were used for *in vivo* experiments in mice and in zebrafish.

Cell culturing

Sf9 (Spodoptera frugiperda) insect cells were maintained at 27°C in TNM-FH (Sigma) medium supplied with 10% FBS, 100 U/ml penicillin and 100mg/ml streptomycin. Phoenix-Ampho and MDCKII cells were cultured in humidified CO_2 (5%) thermostate at 37°C, in DMEM (Sigma) medium supplied with 10% FBS, 100 U/ml penicillin and 100mg/ml streptomycin.

Expression of S1121W, T1301I, Q1347H, R1459C and Δ(2-275)ABCC6 variants in Sf9 cells

10^5 or $5x10^4$ Sf9 (Spodoptera frugiperda) insect cells were co-transfected with linearized baculovirus vector (60ng) and pAcUw21L vector (250ng) containing ABCC6 wt or the mutant cDNA constructs, on 24 well plates using the $Ca_3(PO_4)_2$ precipitation method as recommended by the manufacturer (BaculoGold kit (BD Biosciences Pharmingen). In order to achieve high virus titer the supernatant of the cells was harvested and new cells were sequentially transfected in multiple steps. Viral-supernatants corresponding to the highest expression levels of the recombinant proteins detected by immunoblot were cloned with end point dilution method.

Transfection of Sf9 cells with high-titer recombinant baculovirus

$3x10^7$ cell were transfected with cloned, amplified virus supernatant on 175cm^2 cell culture flasks in a final volume of 9 ml for 1-1.5 hours. Cells were supplemented with fresh media in a final volume of 25 ml and were cultured at 27°C. Cells overexpressing recombinant proteins were harvested after 72 hours.

Membrane preparation

$3x10^7$ cells were harvested 72 hours post-infection and washed with ice cold washing buffer containing 50mMTris, 300mM D-mannitol and 50 µg/ml PMFS, pH 7.0 two times sequentially. Cells collected with centrifugation were resuspended in 4ml TMEP solution: 50mM Tris, 50mM D-mannitol, 20mM EGTA, 2mM DTT, 8 µg/ml aprotinin, 10 µg/ml leupeptin and 50 µg/ml PMSF, pH7.0, and homogenized in Potter-Elvehjem tissue grinders (Wheaton) for 10 min on ice. Cell debris was centrifuged at 300G for 10min at 4°C, and the supernatant was collected. Homogenization and centrifugation was repeated with the pellet. Supernatant was collected and ultracentrifuged for 60.000G for 60 min at 4°C. Membrane pellet was suspended in TMEP solution to obtain approximately 5-10 mg/ml total protein concentration and subjected for final homogenization in Potter-Elvehjem tissue grinders (Wheaton) for 10 min on ice. Aliquots of membrane preparation samples were stored at -70°C until further use.

Cholesterol loading of ABCG2 expressing Sf9 membrane vesicles

During membrane preparation, prior to the ultracentrifugation step, ABCG2 expressing membrane samples were incubated with 2-4 mM choleserol RAMEB cyclodextrin for 20min at 4°C. Cyclodextrin complexes were removed by dilution (20x) and subsequential ultracentrifugation, 60.000G for 60min at 4°C [Telbisz et al, 2007]. Following steps of the membrane preparation were done as described above. Membrane aliquots were stored at -70°C.

Determination of total protein content

1.) Total protein content of the membrane preparation samples was measured with the *modified Lowry-method*. 5µl of membrane preparation samples were diluted in 1.5ml fresh prepared Lowry-reagent (0.01M NaOH, 2%Na-tartrate and 0.5%$CuSO_4$ in 98:1:1 ratio). After adding 150µl of 1N Folin-Ciocalteu's phenol reagent (Sigma) absorbance was measured at 45min incubation time at 660nm. Calibration samples containing 5, 10, 25, 50, 75 µl of 1mg/ml BSA were prepared parallel to each measurement.

2.) To measure total protein content of cell lysates stored in disaggregating buffer (DB: 50mM Tris-PO_4 pH6.8, 2% ß-mercaptoethanol, 2mM EDTA pH6.8, 20% glycerol, 0.02% bromphenol blue) prior to the steps of the *modified Lowry-method* 5 µl of the samples was diluted in 2ml distilled water and solubilized by adding 20 µl of 2% deoxycholate. After 15 min incubation at room temperature protein content was precipitated by adding 750 µl

25% trichloroacetic acid. Samples were centrifuged with 21.000G for 15 min at 4°C and the precipitates were subjected for modified Lowry-measurement. Calibration samples containing 5, 10, 25, 50, 75 µl of 1mg/ml BSA were prepared parallel to the samples from the beginning.

Leammli SDS-polyacrylamide gel electrophoresis and immunoblot

Membrane preparation and cell lysates samples were diluted in disaggregating buffer (DB: 50mM Tris-PO_4 pH 6.8, 2% SDS, 2% β-mercaptoethanol, 2mM Na-EDTA pH 6.8, 20% glycerol, 0.02% bromphenol blue). 1-10µg and 20-40 µg were loaded on 6-7.5% polyacrylamide gel with Protean electrophoresis equipment (Bio-Rad), respectively. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes by standard electro-blotting with Mini-Trans-Blot or by semidry blot equipments according to the manufacturer's protocols (Bio-Rad). ABCC6 wt and mutant proteins were detected with HB6 polyclonal (1:2000) or M6II-7 monoclonal (1:500) antibodies. HRP-conjugated secondary antibodies (1:20000 anti-rabbit and 1:7500 anti-rat) and a chemiluminescent (ECL) imaging (GE Healthcare Life Sciences) was used to develop signals.

Synthesis and purification of the [^{3}H]K3GS conjugate.

The synthesis protocol described in [Chang et al., 1992] has been modified for micro-synthetic conditions. 30 µl solution of 280µM GSH (solved in 10mM DTT) has been prepared by mixing [^{3}H]GSH (36.6 Ci/mmol; Perkin Elmer) and GSH (Sigma) and rapidly added to 0.25 ml of 0.1M K3 (Sigma) solved in ethanol. The reaction was incubated on 4°C overnight in silanized glass tubes. Crystals were vacuum dried and solved in 5% trifluoric acetate. [^{3}H]K3GS conjugate was purified by HPLC on a Teknokroma TR-011349 column (Nucleosil C18, 250x4.6 mm, pore size: 100Å, particle size: 5 µm) and eluted with a water–acetonitril gradient in 0.1% trifluoro-acetic acid. Crystals, recovered after liophylization, were solved in 5%TFA and the chemical identity of the product was verified by HPLC-MS by Szabó Pál in the MTA KKI Ms Laboratory. Unlabeled VK3GS has been synthesized as described in [Chang et al., 1992] and purified by chrystallization and sequential chloroform-washing. The chemical identity of the compound was verified by HPLC-MS by Szabó Pál in the MTA KKI Ms Laboratory. VK3GS solutions were stored at -20°C in dark glass vials and used within 30 days.

^{45}Ca Transport measurements

The relative amount of uptake-competent inside-out vesicles in different membrane preparation samples was estimated on the bases of ^{45}Ca uptake by endogenous Ca-transporters in Sf9 cells. ^{45}Ca transport was performed using rapid filtration. Membrane samples containing 100µg of membrane proteins were incubated at 37°C for 3 and 6 min in a reaction buffer containing 60mM KCl, 35mM HEPES (pH 7.2), 2mM $MgCl_2$, 40 mM K_3PO_4 (pH 7.2), 100µM $^{45}CaCl_2$. ATP-dependent transport was calculated from data measured in the presence and absence of 0.8 mM MgATP. The reaction was started by adding ATP and it was stopped by diluting the reaction with ice-cold washing buffer (100mM KCl, 35mM HEPES (pH 7.2), 1mM $CaCl_2$) and rapid filtration through 0.45µm pore size nitrocellulose filters (Millipore). Filters were washed with 5ml of ice cold washing buffer for two times. Radioactivity was detected in OptiFluor scintillation cocktail (Perkin Elmer) with a scintillation counter (Wallac 1409 DSA).

[^{3}H]LTC4, [^{3}H]E_2-17G, [^{3}H]MTX and [^{3}H]K3GS Transport measurements

Measurements were performed with the same method as described for ^{45}Ca transport. Membrane samples containing 100µg of membrane proteins were incubated at indicated reaction temperatures (27, 37°C) for 0.5-10 min in reaction buffer containing 6mM $MgCl_2$, 40mM MOPS-Tris pH 7.0, 4mM KCl, 2mM DTT. ATP-dependent transport was calculated from data measured in the presence and absence of 4 mM MgATP. In case of where the affect of multiple compounds were studied, these were administered during the preincubation time. The reaction was started with the addition of the radioactively labeled substrates. Chemical concentration was set up by mixing the radioactively labeled and the unlabeled forms of the same compound. The reaction was stopped by diluting the reaction with ice-cold washing buffer (40mM MOPS-Tris pH 7.0, 70mM KCl) and by rapid filtration through 0.45µm pore size nitrocellulose filters (Millipore). Filters were washed with 5ml of ice cold washing buffer for two times. Radioactivity was detected in OptiFluor scintillation cocktail (Perkin Elmer) with a scintillation counter (Wallac 1409 DSA). Transport kinetics were calculated and visualized with KaleidaGraph (Synergy) Software.

Retroviral expression of ABCC6 mutants in MDCKII cells

$7.5x10^5$ Phoenix-Ampho "packaging" cells/well were co-transfected with the recombinant retroviral vector pSpSldS containing the wild type or mutant constructs of ABCC6 cDNA parallel with helper vectors expressing M57 and GALV viral proteins on 6-

well plates. Transfection was performed using the calcium phosphate precipitation method, according to the manufacturers protocol (GIBCO). 12 hours after transfection cells were supplied with fresh medium. Virus containing supernatant was collected 48 and 72 hours post-transfection. $5x10^4$ MDCKII cells were transduced with the previously harvested recombinant retroviruses diluted in DMEM medium (1:1) on 24-well tissue culture plates. Polybrene was also added in a final concentration of 6 mg/ml. After centrifugation (1000G, 90min) cells were maintained at 37°C in humidified, 5% CO_2 incubator.

Immunostaining of MDCKII cells and confocal imaging

In order to obtain nonpolarized cell culture $3x10^4$ MDCKII cells/well were maintained in 500µl D-MEM medium on 8-well chambers (coverslips). Samples were subjected for immunostaining after two days. Cells were washed three times in Dulbecco's Phosphate Buffered Saline (DPBS: PBS (137mM NaCl, 2.7mM KCl, 10mM $Na_2HPO_4 \bullet 2H_2O$, 2mM KH_2PO_4 pH7.4) supplemented with 1mM $CaCl_2$ and 0.5 mM$MgCl_2$) and fixed with 4% paraformaldehide for 5 min at room temperature. After washing in DPBS three times samples were treated with pre-cooled methanol for 5 min. After three washing steps with DPBS samples were incubated in blocking buffer (2 mg/ml BSA, 0.15% Triton X-100, 5% goat serum, 1% fish gelatin solved in DPBS) for 60min. After blocking samples were incubated with primary antibody in appropriate concentration solved in blocking buffer for 60-120min at room temperature. After three washing steps (5 min each) samples were incubated with secondary antibody in appropriate concentration solved in blocking buffer for 60min at room temperature. After three washing steps in DPBS samples were stained with DAPI (1µM) for 5min, if it was indicated. Following final washing steps samples were subjected for confocal microscopy.

To obtain polarized cultures of MDCKII, 10^5cells/well were maintained on Transwells in 250/500µl D-MEM culture media. Cells were subjected for immunostaining 8-10 days after reaching confluency. During these 8 days cells were firmly washed with D-MEM media to remove the attached cell clamps from the surface of the monolayer. Cells were immunostained according to the same procedure as described above for nonpolarized cells.

Confocal imaging of immunostained MDCKII samples was performed using Olympus IX-81/FV500 laser scanning confocal microscope; images were analyzed by Olympus FluoView 4.7 software.

V. RESULTS

V.1. Testing VK3GS as substrate candidate for ABCC6 ABCC1, ABCC2, ABCC3 and ABCG2 hepatic transporters *in vitro*

In the following experiments we primarily raised the question, whether ABCC6 is able to transport the VK3GS compound in cell free transport assay. As VK3GS is the GSH conjugated form of a toxic VitK intermedier, present in the liver, ABCC1, ABCC2, ABCC3 and ABCG2 hepatic transporters were also selected for the study. All of these transporters were previously shown to be involved in the transport mechanism of glutathione conjugated metabolites *in vitro* [Jedlitschky et al, 1994; Leier et al, 1994; Loe et al, 1996; Deeley et al, 2006; Oude et al, 1989 Paulusma et al, 1999; Zeng et al, 2000; Zelcer et al, 2001]. The following transport experiments were performed on membrane vesicles prepared from Sf9 insect cells expressing high amount of the above proteins. Protein content of the samples was determined using the modified Lowry method. The expression level of wild type ABCC1, ABCC2, ABCC3, ABCC6, ABCG2 proteins and the ABCC1 G771D mutant form of the protein was detected by immunoblot, see Fig.17.

ATP dependent uptake of the radioactively labeled VK3GS was measured by rapid filtration method using "inside out" membrane vesicles. Here, nucleotide-binding domains face the outer surface of the memebrane vesicles, and in the presence of ATP, the transporter accumulates the substrate inside the sealed vesicle. Transport rates are calculated from radioactivity values measured in the presence of 4mM MgAMP and 4mM MgATP. The relative amount of inside-out vesicles of membrane preparation samples was estimated by measuring the $^{45}Ca^{2+}$ uptake of endogenous Ca^{2+} transporters. In case of ABCG2 cholesterol loaded vesicles were prepared based on the method described in [Telbisz et al, 2007].

Prior to the VK3GS transport assays membrane vesicles containing high amount of ABCC1, ABCC2, ABCC3, ABCC6 and ABCG2 proteins were validated in control experiments using previously reported substrate molecules [Bakos et al, 1998; Bodo et al, 2003; Iliás et al, 2002; Telbisz et al, 2007]. The background activity of a control membrane, prepared from beta-galactosidase expressing cells, was subtracted from the activity values in each case. Measured data were as follows: ABCC1: ~60 pmol LTC_4/mg

membrane protein/min (50nM LTC_4, 23°C, 0.5 min); ABCC2: 1.6-3.2 nmol E_2-17-beta-G/mg membrane protein/min (100μM E_2-17-beta-G, 37°C, 2 min); ABCC3: 60-70 pmol E_2-17-beta-G/mg membrane protein/min (10μM E_2-17-beta-G, 37°C, 2 min); ABCC6: ~11 pmol LTC_4/mg membrane protein/min (50nM LTC_4, 37°C, 0.5 min); ABCG2: 400-600 pmol MTX/mg membrane protein/min (100μM MTX, 37°C. 5 min). These values are comparable with those published indicating that the transporters are active.

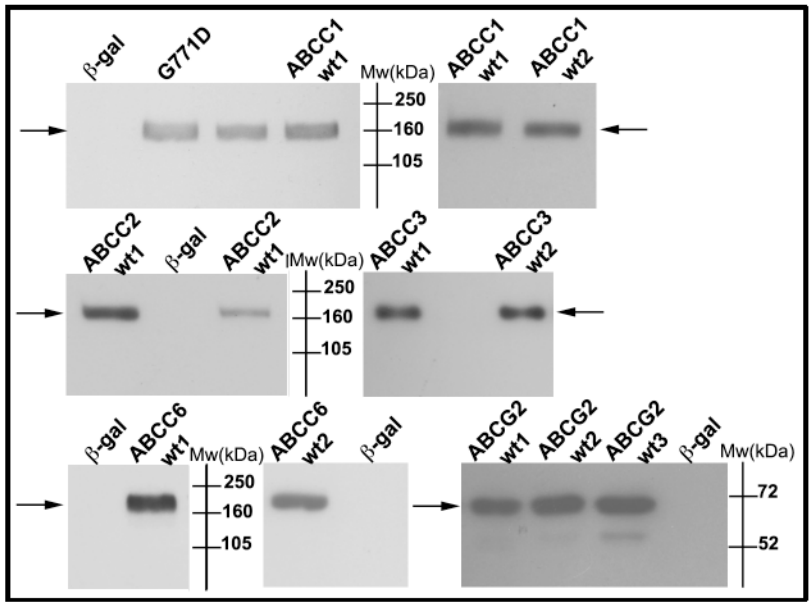

Fig.17: Immunoblot of ABCC1 and ABCC1 G771D, ABCC2, ABCC3, ABCC6 and ABCG2 proteins as expressed in Sf9 insect cells. Sf9 insect vesicles prepared from beta-galactosidase expressing cells were used as negative control. Recombinant proteins were detected by m6 monoclonal, m_2I.-4 monoclonal, m_3II.-9 monoclonal, HB6 polyclonal and BXP-21 monoclonal antibodies respectively. Arrows indicate the expected molecular weight of the proteins.

I have synthesized and crystallized radioactively labeled VK3GS using [^{3}H]GSH and K3 with the method described in [Chang et al, 1992] modified for micro-synthetic conditions. [^{3}H]K3GS was purified by HPLC. Non-labelled VK3GS was generated on the same way. The synthesis has been verified by HPLC-MS by Szabó Pál in MTA KKI Ms Laboratory.

In the first set of experiments transport capacity of ABCC1, ABCC2, ABCC3 and ABCG2 transporters was measured at 60nM and 5μM concentration of [^{3}H]K3GS as it is shown on Fig.18. G771D transport inactive mutant of ABCC1 protein has been used as negative control [Szentpétery et al, 2004]. In case of ABCC6 VK3GS transport rates were

measured at 60nM, 1 and 10 μM concentration points, see fig18. Based on the preliminary experiments we expected a very low transport capacity of the ABCC6 expressing membrane. To achieve stronger data, we have extended the incubation time of the transport reaction to 5 minute. Transport was not in linear phase at 5 minute, thus I present the data of ABCC6 transporter in pmol/mg/5min formula on Fig.18.

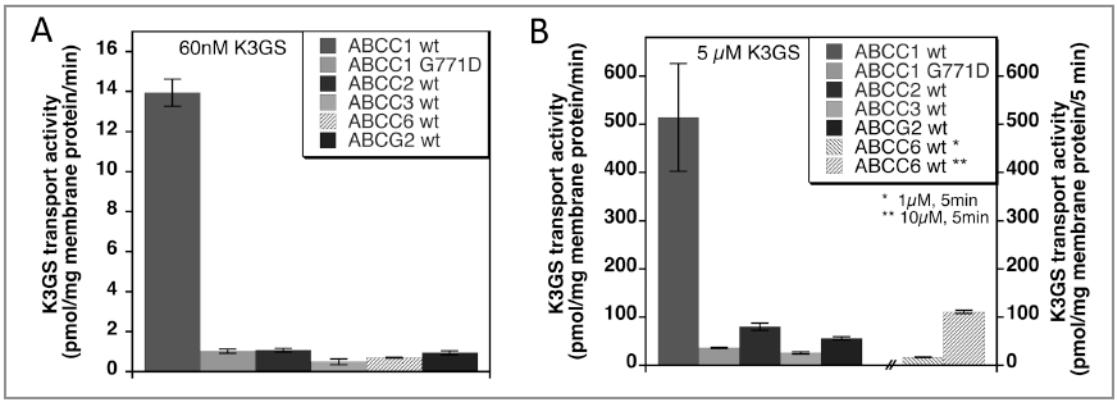

Fig.18. K3GS transport rates of ABCC1, ABCC2, ABCC3, ABCC6 and ABCG2 hepatic transporters. The G771D inactive mutant of ABCC1 protein has been used as a negative control. Two concentrations, 60nM and 5μM of K3GS were used, except in case of ABCC6 protein where transport values were detected at 60nM, 1 and 10μM conditions (see panel A and B respectively). Transport rates were detected after 0,5 min of incubation at 37 °C, except ABCC6 protein, where the 1 and 10μM data were collected after 5 min of incubation time. Data represents the results of transport experiments performed on vesicles originating from two independent membrane preparations.

At low concentration level of K3GS (60nM) we could detected a high transport rate in case of ABCC1 wild type transporter while all the other investigated ABC proteins showed no activity as their transport rates were similar to that of the negative control membrane ABCC1 G771D. The transport rate of ABCC6 protein expressing vesicles was very low at every concentration point of VK3GS compared to ABCC1. Most probably VK3GS is not the physiological substrate of ABCC6 transporter, or at least the transport could not be characterized in this experimental system. However 20μM simultaneously added K3GS conjugate slightly inhibited the LTC_4-transport activity of ABCC6 protein to 78%, see Fig.19, panel A.

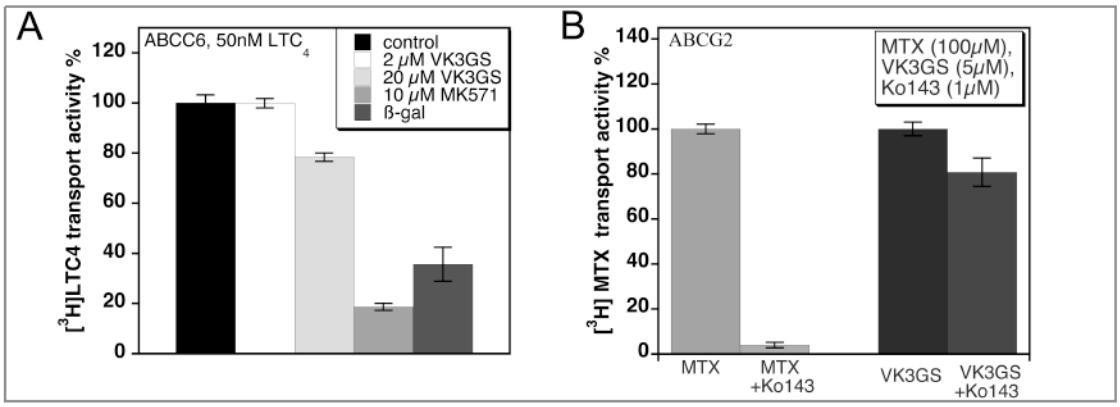

Fig.19: Interactions of VK3GS/LTC4 substrates in transport experiments of ABCC6 protein (panelA); effect of Ko143 specific inhibitor on K3GS transport of ABCG2 protein (panelB). PanelA: Inhibition of ATP dependent LTC_4 transport of ABCC6 expressing vesicles by K3GS and 10uM of MK571 MRP inhibitor. Vesicular uptake was measured at 50nM concentration of LTC_4 at 37°C for 0.5 min. The amount of K3GS added during the experiments is indicated on the inserted panel. Panel B: Effect of 100μM Ko143, the specific ABCG2 inhibitor on the K3GS transport rate of ABCG2. Experiments were performed at 5μM concentration of VK3GS for 5 min at 37°C. Effective inhibition of MTX transport by 1μM Ko143 (control experiment) is also indicated in light gray.

At 5 μM concentration of K3GS ABCC2 and ABCG2 transporters showed a marginal activity. In case of ABCG2 membrane, this transport effect was proved not to be ABCG2 specific, since it was resistant to the specific inhibitor Ko143, see Fig.19 panel B. I measured 80.9% residual transport activity after incubation with 1μM Ko143. In contrast, MTX transport activity of the same membrane could be inhibited with 1μM Ko143 to a residual activity of 3.9%.

The transport activity of ABCC2 containing membrane observed at 5μM VK3GS has been investigated further. It has been shown previously that organic anions can modulate the vesicular uptake of the characterized substrate molecule: $E_2$17βG [Bodo et al, 2003; Zelcer et al, 2003]. Indomethacine in concentrations between 50 and 100 μM induced a 6-6.5–fold stimulation of the $E_2$17βG transport of ABCC2 protein [Bodo et al, 2003 and 2003b]. The stimulatory effect was most prominent at low concentrations of $E_2$17βG and 100μM of indomethacine [Bodo et al, 2003 and 2003b]. In our experiments, 100μM indomethacine significantly increased the transport rate of ABCC2 at 60nM concentration of VK3GS, see Fig.20 panel A. This stimulatory effect at 60nM and the marginal transport observed at 5μM of K3GS supports that VK3GS is a substrate candidate for ABCC2 hepatic transporter, and that ABCC2 is probably a low capacity transporter of K3GS

conjugate. In case of $E_2$17βG indomethacine was proved to be a strong allosteric activator [Bodo et al, 2003]. Further experiments in a wide concentration scale of both compounds, VK3GS and indomethacine, could elucidate whether the same allosteric effect was the background of the stimulatory effect of 100μM IM observed at 60nM of VK3GS.

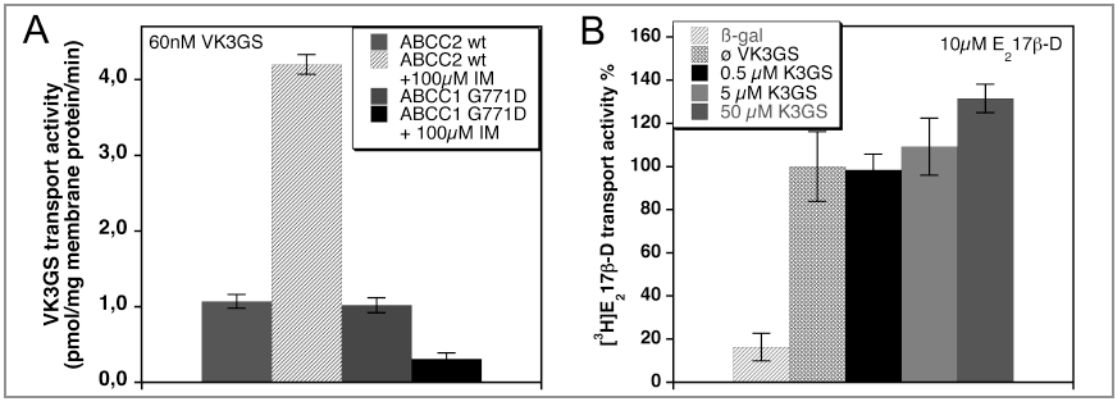

Fig.20: Interactions of IM/VK3GS and VK3GS/$E_2$17G substrates in transport experiments of ABCC2 protein. PanelA: The effect of simultaneously added IM on the K3GS transport of ABCC2 transporter. Experiments were performed at 60nM concentration of K3GS and 100 μM IM for 0.5 min at 37°C. PanelB: The effect of simultaneously added K3GS conjugate on the vesicular uptake of $E_2$17G by ABCC2 protein. Experiments were performed at 10μM concentration of E_2-17G and 0-0.5-5-50 μM K3GS for 2 min at 37°C.

Since the amount of radioactively labelled VK3GS was very limited I could not start large scale experiments to study the effect of indomethacine on the VK3GS transport. Furthermore, I investigated, whether VK3GS has an effect on the [^{3}H]E_2-17-beta-G transport of ABCC2. Interestingly, simultaneously added K3GS conjugate in a concentration range of 0.5-50 μM did not alter the E_2-17G transport of ABCC2 significantly, see Fig.20 panel B.

As I have measured a relative high rate of VK3GS transport in case of ABCC1 transporter, I decided to investigate the characteristics of this transport. VK3GS transport of ABCC1 was essentially linear up to 1min (see Fig.21 panelA), which enabled to determine the kinetic parameters of the transport at 0.5 min. In my experiments I revealed 1.45μM approximate K_M and 240 pmol/mg membrane protein/min V_{max} values of ABCC1 protein for VK3GS at 23°C (see Fig.21 panel B). These values are comparable to the transport rates of ABCC1 for LTC4 and NEM-GS substrates (LTC4: Km 135±35 nM, Vmax 200±20 pmol/mg/min NEM-GS: Km 155±40μM, Vmax 580±60 pmol/mg/min)

[Bakos et al, 1998] that might indicate a physiological role for the ABCC1 transporter in K3GS transport.

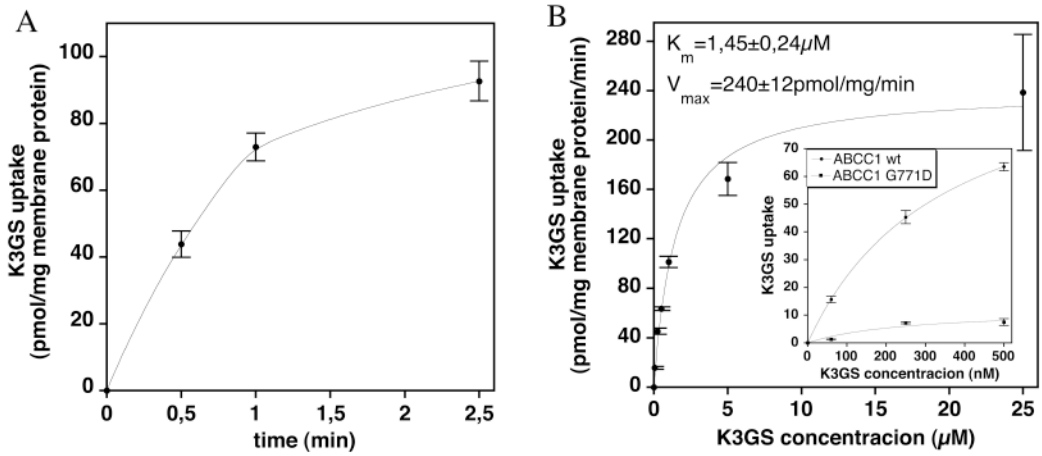

Fig.21: K3GS uptake kinetics of ABCC1 transporter. Panel A: timecourse of K3GS transport of ABCC1 expressing Sf9 insect vesicles. Transport was measured in the presence of 500nM K3GS at 23°C. Panel B: concentration-dependence of K3GS uptake of ABCC1 expressing Sf9 insect vesicles. The rate of transport was measured after 0.5 min incubation on 23°C in a concentration range of 60nM to 25μM. The transport rate of G771D inactive mutant (♦) compared to wild type (●) at 60, 250 and 500 nM concentrations is shown on the insert of panel B.

I also investigated the modifying effects of VK3GS and LTC_4 substrates in transport measurements when these substrates were introduced simultaneously. In parallel experiments the effect of unlabeled LTC_4 conjugate on the transport of [^{3}H]VK3GS and vice versa was investigated at given concentrations. Since the amount of the radioactively labeled VK3GS was very limited the effect of LTC_4 on the transport of [^{3}H]VK3GS was investigated only at one experimental condition: 150nM of [^{3}H]VK3GS, 500nM of LTC_4 at 23°C. Experimental conditions were selected based on the results of Éva Bakos [Bakos et al, 1998]. 500nM simultaneously added LTC_4 inhibited the ABCC1-K3GS transport activity to 35 %, see Fig.22 panel A, columns on the left. 2-5μM K3GS did not effect the [^{3}H]LTC4 transport activity of ABCC1 while 25 μM K3GS inhibited the transport to 56 % when measured at 50 nM of [^{3}H]LTC_4, at 23°C, see Fig.22 panel B.

The effect of the general MRP inhibitor MK571 was also measured. 10μM MK571 inhibited the ABCC1-VK3GS transport activity to 23 %, see Fig.22 panel A, columns on the right.

These above results proved that the K3GS transport of ABCC1 is specific; it was ATP dependent, the inactive mutant G771D did not show any activity, simultaneously introduced MK571 MRP inhibitor or LTC4 physiological substrate both inhibited the K3GS transport of ABCC1.

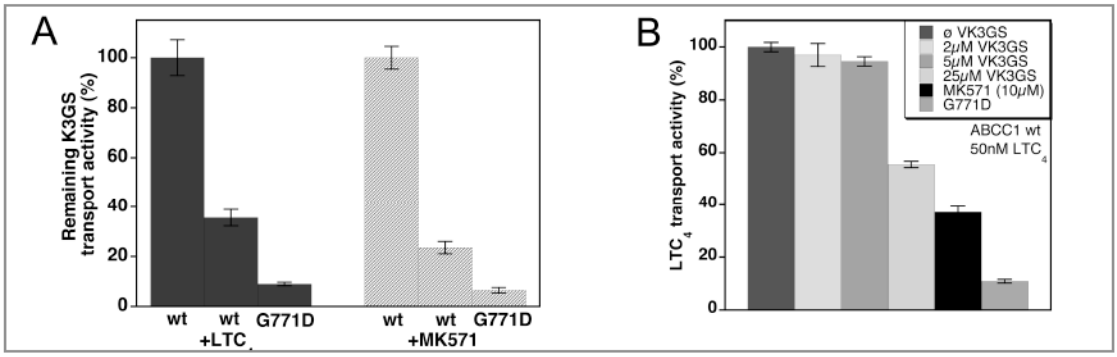

Fig.22: Interactions of VK3GS and LTC4 ABCC1 substrates in transport experiments and the effect of MK571 inhibitor on VK3GS transport of ABCC1 protein. Panel A: Inhibition of ATP-dependent VK3GS transport of ABCC1 expressing Sf9 insect vesicles by LTC_4, a well-known ABCC1 substrate (columns on the left) and the MK571 MRP-inhibitor (columns on the right). Transport rates were measured in the presence of 60 nM K3GS and +/- 500nM LTC_4 at 23°C for 0.5 min and 150nM K3GS +/- 10μM MK571 at 37°C for 0.5 min respectively. Panel B: Inhibition of ATP dependent LTC_4 transport of ABCC1 expressing vesicles by VK3GS. Experiments were performed in the presence of 50 nM LTC_4 and 0-2-5-25 μM K3GS at 23°C for 0.5 min.

In summary, *in vitro* transport experiments revealed ABCC1 as a high capacity transporter and ABCC2 as a low capacity transporter of the VK3GS metabolite. No transport of the K3GS compound was detected for ABCC3 and ABCG2 hepatic transporters.

I emphasize that the main objective of our experiments was to elicit, whether ABCC6 is the hepatic transporter of VK3GS metabolite. We could not detect considerable transport activity of ABCC6 protein for the [^{3}H]VK3GS substrate while the transporter was active in the very same assay for [^{3}H]LTC4 test substrate. I conclude that most probably ABCC6 does not transport VK3GS in the liver, i.e. VK3GS is not the physiological substrate of the transporter.

These results were in harmony with *in vitro* vectorial transport experiments performed by our collaborative partners in the Netherlands Cancer Institute, Amsterdam. No significant difference compared to control MDCKII cell was observed in case of ABCC6

overexpressing cells, while ABCC1 overexpressing cells showed a remarkable increase in apical–to-basal efflux relative to control cells [Fülöp et al, 2011]. *In vivo* liver perfusion experiments of wild type and *Abcc6-/-* mice, performed parallel in our laboratory by Viola Pomozi and by collaborative partners in the Thomas Jefferson University, Philadelphia, also supported our findings. VitK3 was administered into the portal vein of mice. The glutathione conjugated form of VitK3 (VK3GS) in the outflow fluid of the inferior vena cava was detected only when VK3 was added before (HPLC-MS assay of K3GS was performed by Pál Szabó, MTA KKI.). No significant difference in the VK3GS levels of the outflow fluid was detected between wild type and *Abcc6-/-* mice [Fülöp et al, 2011]. In harmony with these results excessive amounts of vitamin K used in feeding experiments of the Abcc6 -/- animals did not counteract the ectopic calcification [Jiang et al, 2011; Brampton et al, 2011; Gorgels et al, 2011]. All these above findings argue against the role of ABCC6 in the hepatic transport of K3GS metabolite and suggest that PXE is not a direct consequence of vitamin K deficiency [Fülöp et al, 2011; Jiang et al, 2011; Uitto, 2014].

V.2.1. ABCC6 sequence variation database:

Since 2000, when mutations at the *ABCC6* locus were identified as the genetic background of PXE [Bergen et al, 2000; LeSaux et al, 2000; Struck et al, 2000; Ringpfeil et al, 2000], more than 400 sequence variations affecting this region (NCBI Reference Sequence: NG_007558.2; Ensembl: ENSG00000091262.10) were published. Besides the numerous single nucleotide substitutions large deletions, eliminating several exons or even larger ones, extending across the genomic region of *ABCC1* and *ABCC6* genes were also described. Genetic mutations affecting the coding region, especially amino acid substitutions or small deletions, with known pathogenicity can largely support structure and function approaches of a protein. The large number of disease associated mutations and sequence variations were no longer manageable by searching and reading the literature every time.

In 2007, together with Orsolya Symmons we established an ABCC6 sequence variation database in our laboratory. Our aim was to collect all the sequence alterations reported thus far. We thoroughly searched the literature and collected 50 papers reporting on 300 sequence variations, affecting both the coding and the non-coding regions of *ABCC6* gene. Sequence variants found in the pseudogenes, *ABCC6ϕ1* and *ABCC6ϕ2,* were also taken.

These data were involved in Orsolya's work focusing on *ABCC6* gene evolution and are not presented in this thesis. Data processing of the *ABCC6* gene was done by both of us. In 2012 it was reported that mutations in *ABCC6* gene are also responsible for a subset of GACI (Generalized arterial calcification of infancy) cases [Nitschke et al, 2012; Li et al, 2013a and 2013b]. Most GACI patients carry mutations in the Ectonucleotide Pyrophoshatase/Phosphodiesterase 1 (ENPP1) gene. GACI patients present calcification phenotype that is very similar to the characteristics of PXE, although the disease is more severe. The mutation spectra of ABCC6 in PXE and GACI overlap. GACI mutations represent only a minority of ABCC6 mutations. These data are also included in our table. From time to time database was updated; now (08/10/2013) the full table lists 429 potential sequence variations as referred in 63 papers. During our work we paid special attention to revise the viability of the methods that were used for sequence analysis in the papers. Particularly, whether these techniques were selective enough to distinguish between the sequence of *ABCC6* gene and those of the two pseudogenes. In case of 73 entries of the database we found potential errors in the reported mutations or sequences. In order to revise data we have contacted the corresponding authors in email. For a representative formal letter see supplementary material, supp.fig4. To date, the number of corrected (confirmed) mutations is 414. These unambiguously described sequence variations, are listed in supp.table1. Database has been last updated in July 2013. Summary tables representing the database are attached to my thesis as supplementary material, see supp.tabels 1-7.

Information listed in the columns of the database:

Column A: "sequence variation at DNA level": In this first column sequence variants are described at DNA level, in a format that is recommended by the HGVS (Human Genome Variation Society), see column A of supp.table1. For a detailed description of the HGVS nomenclature recommendations see webpage: http://www.hgvs.org/mutnomen/recs.html. Briefly: e.g. "c.619G>A" indicates a single nucleotide substitution of a G for an A at the nucleotide position 619 of the cDNA; "c" stands for coding DNA sequence, used as reference. In case of deletions or insertions the first and last affected nucleotide is indicated, they are separated by mark "_", and followed by the abbreviation: "del" or "ins". Nucleotide positions that are in the non-coding region are numbered according to the position of the first or last nucleotide of the most proximate

exon. The 5' or 3' direction of the affected position is signed by "-" or "+" respectively and the distance is also given. For ABCC6 reference sequence see: ABCC6 cDNA Reference Sequence (NCBI Reference Sequence: NM_001171.5). The "A" in the triplet of the initiator methionine is denoted as nucleotide position "1".

Column B: "Sequence variation at protein level": In case of sequence variants that affect the coding region, the alteration at protein level is reported here. The "p." stands for protein, "del" for deletion, "ins" for insertion and "fs" for frameshift. The one-letter symbol abbreviations of the amino acid residues are given as recommended by IUPAC (http://www.iupac.org/publications/pac/1984/pdf/5605x0595.pdf). The initiator methionine is denoted as position "1".

Column C: "Mutation/polym.": The status, mutation or polymorphism, of a sequence variant, as it was concluded by reading all papers reporting on it, is indicated in "column C". "M" or "P" stands for mutation or polymorphism respectively. We have reviewed the papers by the reliability of the methods that have been used to determine the status of a sequence variation: mutation or polymorphism. The official directive upon a variation can be referred as a mutation or a polymorphism was concluded in [Cotton and Scriver, 1998]. Briefly, it is as follows: A.) Sequence variants resulting in nonsense or splice-site alteration can be designated as disease causing if they co-segregate with the disease haplotype in affected (but not in unaffected) members of the families with the disease; and if they were not detected in 200 control alleles of unrelated and unaffected individuals. B.) Nucleotide variations that result in amino acid substitution are termed disease-causing when: 1.) The variant co-segregates with the disease phenotype and haplotype in all PXE pedigrees carrying it, 2.) No other potentially disease-causing and familial haplotype-specific allelic variant was identified by complete sequence analysis of the gene, 3) the variant was not found in the cohort of 200 control chromosomes, and 4) the amino acid substitution involved a conserved amino acid.

The total number of mutations collected in our table is 220 (see supp.table 2.), the total number of polymorphisms, reported by the papers and the HapMap database, was 154 (see supp.table 3.). A more detailed description of the categories of mutations and polymorphism is given later in the section of "column D". In case of 40 variants published data was inconclusive, thus the status could not be determined. These latter variants are labelled with marks: P?, M? or P/M (see supp.table1.).

Column D: "Type": The type of the alterations is described in this column. Abbreviations given in this column are as follows: del/deletion; ins/insertion; dup/duplication, fs/frameshift; n/nonsense, m/missense or s/samesense for the amino acid substitution. If a mutation affects the first or last 3 nucleotides of an exon or the first and last 20 nucleotides in the flanking intronic area, it is referred as a potential splice site mutation and it is indicated as "pot.splice" here. Intronic mutations are labelled with blue and if not otherwise, they are indicated with "i". All exonic variants are labelled with red.

We have found 144 **intronic** variants; out of which 11 were concluded as disease causing, these affect exon-intron barriers and are considered as potential splice mutations in the literature. In one case, c.2248-2_2248_1del [Gheduzzi et al, 2004], skipping of exon18 was confirmed by RT-PCR and thus the mutation is referred as p.750_805.

Out of the 246 **exonic** variants, 185 were concluded as disease causing, and 32 as polymorphisms (see supp.table 6.), while in case of 29 the status could not be determined. Published data unambiguously proved 10 single nucleotide substitutions, resulting in non synonymous amino acid replacement, as being polymorphic variations, since they could be isolated from healthy controls as well as from PXE patients and did not co-segregated with the disease phenotype, labelled blue in supp.table 6. All the remaining 22 polymorphic variants result in same sense amino acid replacement (silent variants), see supp.table 6. The distribution of the 185 exonic mutations, associated with the PXE phenotype was as follows: majority of the mutations are single nucleotide substitutions; 124 missense and 23 nonsense alterations, see supp.table 5. We have collected 28 small deletions, 8 insertions and 2 duplications, see supp.table 4., the majority of these would result in frame shift mutations.

Large deletions, spanning several exons and introns, are listed at the end of the supp.table 1. In many cases, it is very difficult to locate them precisely, thus they are referred redundantly in the literature. In our collection we list 23 large deletions that we have found in the papers these are probably not all unique.

Column E: "Genomic pos.": The genomic position of each sequence variation is described in this column based on the genomic position numbering downloaded from Ensembl Genome Browser in July 2013; ENSG00000091262.10.

Column F: "Genomic region affected": This column lists the affected intron, exon or flanking region of *ABCC6* gene.

Column G: "Protein region affected": In case of alterations of the coding sequence the affected protein region or domain is indicated in this column. This regions are predicted by multiple alignment of ABC proteins, by the membrane topology model, based on [Tusnády et al, 1997], or by the homology model [Fulop et al, 2009] of ABCC6 protein, both located to the server of the Institute of Enzymology: http://www.enzim.hu/~varadi/abcc6_topmod.html http://www.enzim.hu/~varadi/ABCC6/

Column H: "C>T at 5'CpG": If a C>T or G>A single nucleotide substitution occurred due to the deamination of a 5-methyl cytosine, since it was originally located to a 5'CpG site, is indicated with a "+" sign in column "H". In vertebrate genomes cytosine residues that are located 5' to a guanosine in coding regions usually are methylated by a methyltransferase. 5-methyl cytosines than spontaneously get deaminated to thymine residues. There is a repair mechanism, which specifically recognizes G-T mismatch sites and replaces T with C. However this repair is not efficient enough and such mutations are frequently observed in heritable diseases [Cooper and Youssoufian, 1988; Holliday et al, 1993]. Most likely for this reason the frequency of CpG dinucleotides in vertebrate genome sequences; with the exception of some regions that escape CpG suppression, e.g. CG-rich promoters; is significantly underrepresented [Bird et al, 1986; Burge et al, 1991; Schorderet et al, 1992].

The GC content of the 4512 nucleotide long ABCC6 cDNA is 60.6%, as it is calculated by the following software: http://www.sciencebuddies.org/science-fair-projects/project_ideas/Genom_GC_Calculator.shtml. Number of cytosines are 1382; number of guanines are 1353. The total number of CpGs along the 4512 base pair coding region is 156 (312 bases involved). The distribution of CpG dinucleotides along the coding region is represented on supp.Fig2.

Amongst the 429 sequence variations that were collected in our work we found 343 single-nucleotide substitutions, 90 (26.24%) of these occurred on 5'CpG sites, see supp.table 7. Now, along the coding region we have found 205 single nucleotide substitutions, out of which 59 (29%) affected CpG sites, see supp.table 7. 48 of these were missense, 6 nonsense and 3 samesense substitutions. In conclusion, it is clear, that the distribution of single nucleotide substitutions along the coding sequence is not random, CpGs are more frequently affected, than other nucleotides. 29 % (59 out of 205) of the reported single nucleotide substitutions affecting the coding region occurred on 312 bases

which correspond to the 6,9 % of the coding nucleotides.

On the other hand, similar to that reported in case of several genetic diseases [Holliday et al, 1993], mutational events affecting CpG dinucleotides largely contribute to the number of disease causing mutations in PXE. Mutations identified on CpG sites along the *ABCC6* gene (45, see supp.table 7.) represent the 20.5 % of all disease causing mutations (220), based on the data reported until 2013. The most frequent disease causing mutation, found in 20-30 % of the PXE alleles [Pfendner et al, 2008], the c.3421C>T; p.R1141X, is also located to a CpG site, as Arg1141 is encoded by the CGA triplet.

Column I: "References (allele frequency data and comments)": This column lists all the articles that report on a given sequence variation. In every case where it was available allele frequency data is also indicated here. Our major comments or conclusions are also present here.

In summary, overall in *ABCC6 gene* we collected 220 disease causing mutations and 154 variations that are described as polymorphisms in the literature. Besides the large number of intronic single nucleotide substitutions, we found 10 missense amino acid alterations and 22 silent amino acid substitutions that are reported as polymorphisms. The 220 PXE associated mutations are as follows: 11 intronic single nucleotide substitutions that most probably cause miss-splicing of the mRNA; 1 small deletion spanning an exon-intron barrier; 23 large deletions; 38 small deletions, insertions or duplications, the majority of these are out of frame mutations; and 147 single nucleotide substitutions, that result in 124 missense and 23 nonsense alterations. The missense and nonsense amino acid substitutions contribute 67 % to the total number of PXE causing mutations.

The online ABCC6 database

In 2008 PXE international, the American advocacy organisation of PXE patients (www.pxe.org), initiated collaboration in order to synthesize our data sets and establish an online open database for ABCC6 sequence variations [Váradi et al, 2011]. Since June 13, 2013 ABCC6 database operates on the server of the NCBI powered by an LOVD platform: http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6. LOVD stands for Leiden open (source) variation database. LOVD provides a flexible, freely available tool for Gene-centered collection and display of DNA variations. After registration database is open for submission of new data. Curators have control over the variants, decides which data are public and curates incoming submissions. LOVD allows for many different ways

to view and browse through the data. Our collection of ABCC6 variants, described in the previous section, served as major basis in establishing the online database.

The membrane topology model of ABCC6 [based on Tusnády et al, 1997, created by László Barna in 2008], see fig. 1A, and the homology model of ABCC6 [Fulop et al, 2009], see fig.1D, described in the following part of the thesis, are also linked to the online database. One can easily visualize the localization of ABCC6 variants in the linear topology or in the three-dimensional model of the protein; conserved regions can be also represented. It is an interactive surface and hence it is a very useful tool in initiating or testing new research ideas related to mutations described in the protein structure.

The additional new feature of the online database, that would not be available without contribution of PXE International, is the validated collection of pathological data of patients with the genetic diagnoses of PXE. Clinical data are concluded according to Phenodex index, which is a validated registry of the phenotypic stages of symptoms described amongst PXE patients. A screenshot of the ABCC6 LOVD homepage is attached to my thesis as supp.Fig 1. Tables that include data collected and catalogued with the help of researchers of our group (Orsolya Simmons, Krisztina Fülöp, Tamás Arányi and András Váradi) are highlighted in orange.

Despite the large number of PXE-associated ABCC6 mutations, published thus far, no clear genotype-phenotype correlation has emerged. Homology model building, via embedding missense mutations in a structural view of the protein, gives an opportunity to answer structural and/or functional consequences of mutations.

V.2.2. ABCC6 homology model:

Model of the closed conformation, based on Staphylococcus aureus Sav1866

Before ABCC6 homology model was generated the only available information about protein folding was the two-dimensional membrane topology prediction of ABCC6, see Fig.23 panel A. Topology model was calculated by Lászlo Barna (Institute of Enzymology, HAS), and it is based on the *in silico* membrane topology analysis of ABCC1, a close homolog of ABCC6 with amino acid identity of 45%. Limited proteolysis supported these models [Bakos et al, 1996; Sinkó et al, 2003]. The three dimensional view of the protein can largely help researchers who are interested in protein structure-function analysis. However, crystal structure data of large transmembrane proteins, like human ABCC6, are

very limited. Homology modelling can serve as useful tool in visualizing the structure of a protein, without any crystal data. One can also visualize the localization of missense mutations in this three dimensional view of the protein. In most cases, missense mutations in recessive disorders, like PXE, lead to the loss of protein function, e.g. by abolishing catalytic activity or by altering protein stability/folding. Accordingly, amino acid residues, which have been observed as being the subjects of disease causing mutations, have large probability to be involved in structural or functional surfaces of the protein. Thus, genetic mutations can serve as tool, in the better understanding of structure and function evidences of the protein. Here I show an analysis, where I have incorporated the information embedded amongst the large number of genetic variations affecting the coding region into the structure predicted by homology modelling.

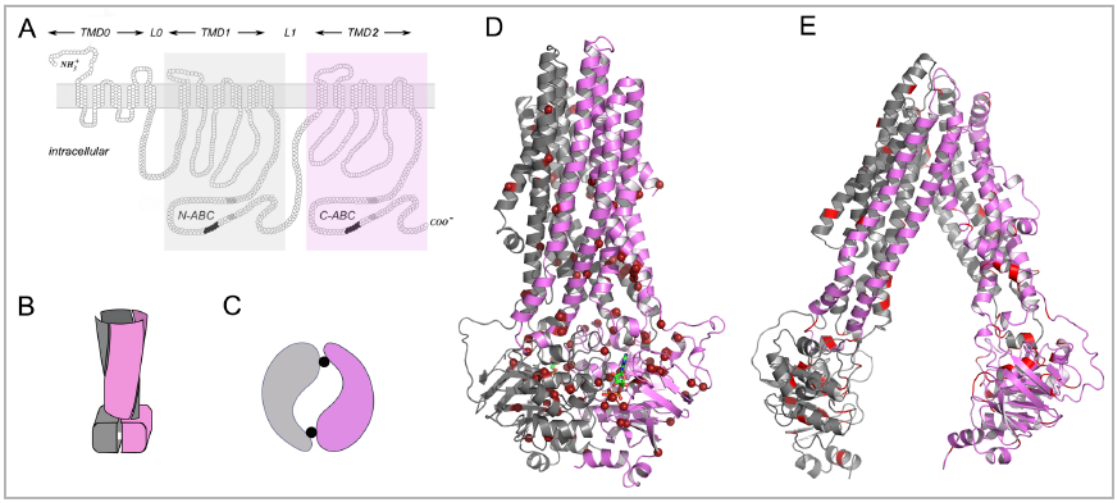

Fig.23: panel A: Membrane topology model of ABCC6 protein. C- and N- terminal regions, that are represented in the models are labelled grey and pink respectively. Grey colour within the linear sequence of ABC folds represents Walker-A and Walke-B motifs, while black indicates the characteristic signature motif of ABC transporters located upstream to the Walker-B motif. Panel B and C: schematic representation of domain swapping (B) and head to tail orientation of nucleotide binding domains (C) in ABC transporters. Panel D and E: Homology models of ABCC6 protein representing closed and open conformation respectively. PXE-associated missense mutationsa are indicated with red.

Based on the crystal coordinates of Sav1866 (PDB ID: 2ONJ, 3.4Å) [Dawson RJ et al, 2006 and 2007], a homodimeric bacterial transporter, we have constructed a homology model of ABCC6 protein, see Fig. 23 panel D [Fulop et al, 2009]. This work was a colaboration with László Barna (Institute of Enzymology, HAS). For better prediction of

ABC domain structures the coordinates of two high resolution ABC–ABC dimers, HlyB and the CFTR [Zaitseva et al, 2004 and Atwell et al, 2007] were also utilized. In the Sav1866 homodimer structure, that represents an outward-facing conformation, corresponding to the ATP-bound state, the two transmembrane domains form a substrate translocation pathway, see Fig.23 panel B and D. The low-affinity binding site of the translocation pathway is exposed to the outer leaflet of the plasmamembrane and the extracellular space; thus the substrate may escape into the outer leaflet or into the surrounding medium of the cell [Dawson et al, 2006 and 2007]. Nucleotide-binding (ABC) domains are in close proximity to each other in harmony with the previously described ‘‘nucleotide sandwich dimer” [Smith et al, 2002]; they are in the characteristic head-to-tail orientation (for a schematic representation, see Fig.23 panel C, for a more detailed representation see Fig.24 panel B and Fig.25 panel B). The Sav1866 structure, published by Dawson et al. in 2006, predicted new, previously unidentified structural elements of the ABC-proteins: the long intracellular loops (ICL). ICLs are ‘‘rigid” extensions of the transmembrane helices, each Sav1866 monomer harbours two. The conformational changes generated during ATP hydrolysis are transmitted to the substrate translocation pathway via the ABC–ICL communication [Dawson et al, 2006]. “Coupling helices” are short helices within ICLs, and are oriented parallel with the membrane plan, and they are located in close proximity to conserved residues in the ABC domain. The “transmission interface” is formed via domain-domain interaction between the four coupling helices and conserved residues in the two ABC domains. A special type of “domain swapping” can be recognized here; the coupling helices contact with the opposite ABC-domains too (for a schematic representation, see Fig.23 panel B).

The homology model of ABCC6, as it was published in [Fulop et al, 2009], is represented on Fig. 23 panel D. The two halves of the molecule are represented in different colours. The first transmembrane domain (TMD0) and the Lo linker, which are characteristic elements of the “long-MRPs”, long-C-type transporters, could not be included in the model, since Sav1866 protein lacks these domains. The same is true for the flexible loop connecting the C- and N-terminal half of ABCC6 protein. Coordinates of ABCC6 homology model are now available at http://www.enzim.hu/~varadi/ABCC6/ .

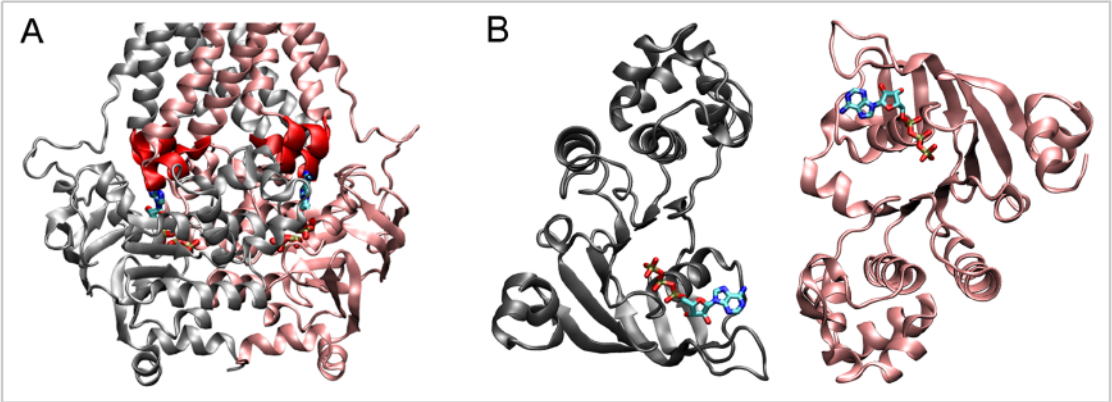

Fig.24: A: Orientation of coupling helices of the interconnecting loops contacting the nucleotide binding domains in the ABCC6 homology model. The four coupling helices are highlighted in red. B: The head to tail orientation of nucleotide binding domains in the homology model of ABCC6 protein. The two halves are opened at the contacting surface by 180°. N-and C-terminal halves of the protein are indicated with grey and pink on both panels.

After ABCC6 model was generated I started to analyse the distribution of PXE-missense mutations on the potential structural surfaces of the protein. During my analysis I have paid special attention to potential domain-domain interactions; the catalytic surfaces formed by the nucleotide binding domains (ABCs) and the ICL-ABC contact surfaces. The validated collection of disease causing mutations, the ABCC6 database described in the previous section, was very beneficial in this work. PXE-mutations were taken from the literature available in December 2008. The number of published mutations that time was 132. I made a careful filtration of this data set and only the unambiguously documented 119 mutations were included into the analysis. These are highlighted on the model on Fig.23 panel C and D.

I have observed significant clustering of PXE-missense mutations on the following two surfaces: 1.) The ABC-ABC contact surface and 2.) The ABC-ICL " transmission interface" formed by conserved residues of ABC domains and the four coupling helices.

Mutations at the ABC-ABC interface

Fig.25 panel B represents the ABC-ABC interface. Amino acid residues that are predicted to be situated within 4Å distance to residues of the opposite ABC domain or to the MgATP nucleotide in the outward-facing conformation of ABCC6 are highlighted in blue. Residues that are affected by disease causing missense mutations are represented with red colour. According to the model 68 amino acid residues were found to be

potentially involved in the above interaction and out of these 19 are affected by mutations described in PXE patients, see table 3. This ratio (19/68) is 3.53 fold higher than the overall mutational frequency (referring to missense mutations) of the molecule (119/1503) ($p<0.0001$), see table 4., indicating that this part of the protein structure is significantly less permissive for missense mutations. These data represents a ”genetic proof” of the essential role of these interacting surfaces.

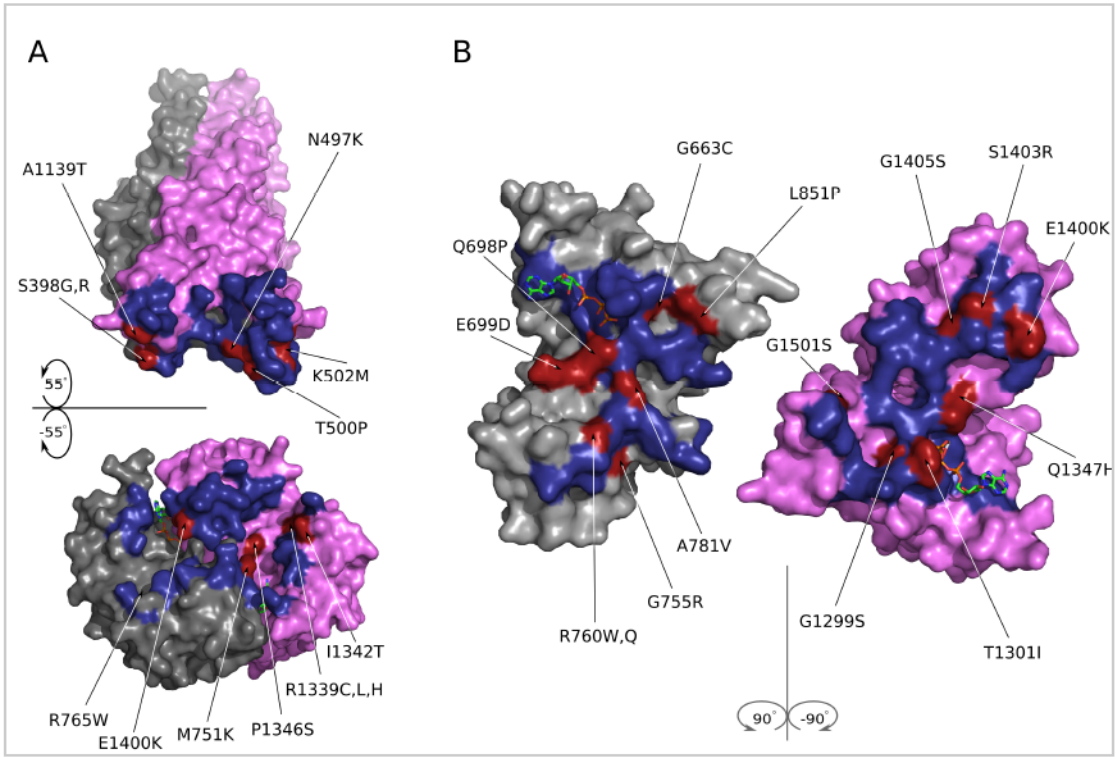

Fig.25: Clustering of missense mutations at the ICL-ABC (A) and ABC-ABC (B) contact surfaces. Residues predicted within 4Å distance are highlighted in blue. Missense mutations identified in PXE patients are indicated on the figures, the corresponding residues are highlighted in red. For better visualization model is opened at the contact surfaces; 110° ABC-ICL, 180° ABC-ABC.

Mutations at the transmission interface

The transmission interface, formed by conserved residues of ABC domains and the four coupling helices of the TMD1 and TMD2 domains oriented within 4Å distance proximity in the predicted structure, is represented on Fig.25 panel A. 69 amino acid residues, that meet the less than 4Å criteria, and thus potentially are involved in the interaction, are labelled with blue. The 15 PXE mutations affecting the surface are highlighted in red. For

a summary table of the contact residues and mutations affecting ABC-ICL surface see table 3. The mutational rate of this area (15/69) is 2.75-fold higher than that of the whole molecule (119/1503)(p=0.0025), see table 4. The high frequency of disease causing mutations suggests, that the ABC-ICL interacting surface is less tolerant to missense substitutions, than the overall ABCC6 transporter. This is in harmony with the finding of Dawson et al, 2006 about the Sav1866 structure, predicting a fundamental role for these domain-domain surfaces in ABC protein function.

ABC-ABC contacts 4.0 Å (contacts with MgATP are also included)	
∑68	638,663,664,665,666,667,668,669,670,671,698,699,752,753,754,755,756, 757,760,777,778,781,782,783,784,786,789,812,813,814,815,851,1274,1299, 1300,1301,1302,1303,1304,1305,1306,1307,1347,1387,1398,1400,1401,1402, 1403,1404,1405,1406,1407,1426,1427,1430,1431,1432,1433,1434,1436,1458, 1459,1493,1497,1500,1501,1503
Mutations ∑19	G663C,Q698P,E699D,G755R,R760Q,R760W,D777N,A781V,L851P,G1299S, T1301I,G1302R,A1303P,S1307P,Q1347H,E1400K,S1403R,G1405S,G1501S

ABC-ICL contacts 4.0 Å in closed conformation	
∑69	398,399,401,402,404,406,489,493,496,497,499,500,502,503,504,505, 506,507,513,638,679,680,701,703,704,713,715,747,748,751,752,765, 769,1033,1034,1037,1038,1039,1040,1041,1129,1132,1136,1137,1139, 1140,1142,1146,1275,1313,1315,1316,1336,1339,1342,1346,1350,1351, 1352,1353,1358,1362,1363,1396,1397,1398,1400,1401,1414
Mutations ∑15	S398G,S398R,N497K,T500P,K502M,M751K,R765W,R765Q,A1139T, R1339C,R1339H,R1339L,I1342T,P1346S,E1400K

ABC-ICL contacts 4.0 Å in the open conformation	
∑41	398,402,493,494,497,500,502,503,504,639,679,699,701,703,713,714,715, 716,769,1034,1037,1038,1126,1132,1136,1138,1273,1274,1310,1313,1315, 1316,1339,1344,1350,1352,1362,1363,1364,1414,1418,
Mutations ∑14	S398G,S398R,N497K,T500P,K502M,E699D,R1138W,R1138Q,R1138P, R1273K,R1339C,R1339H,R1339L,R1418Q

Table 3: Contact residues that are involved in domain-domain interactions investigated in this study. Missense PXE-mutations, identified in patients so far, are also listed in the table. Mutations, that are result of C>T or G>A substitution at a CpG site are highlighted in 'bold'.

These results representing the unequal distribution of PXE associated missense mutation in the predicted ABCC6 structure as well as the description of the homology model itself was published in [Fulop et al, 2009].

ABCC6 homology model of open conformation

In March 2009 the crystal coordinates of mouse Abcb1 (PDB ID: 3G5U, 3.8Å), in the nucleotide-free inward-facing conformation, were published [Aller et al, 2009]. According to this structure the coordinates of the first eukaryotic ABC protein became available. The structure represents an initial stage of the transport cycle; the transporter is competent for drug binding. The nucleotide binding domains (NBDs) are separated by ~ 30Å.

We have repeated model building using the above, inward-facing structure coordinates. For a representative illustration see Fig.23 panel E. Since NBDs are oriented far from each other in this structure, the distribution of mutations could be investigated only in case of the ABC-ICL surface. Similar to our previous findings a 4.31 fold accumulation, as it is represented on table.4, of disease-associated mutations was detected on this contact surface. 14 of the 41 amino acid residues that meet the 4Å criteria are affected by PXE mutations, see table 3 [Váradi et al, 2011].

	residues	mutations	ratio	fold bias
ABCC6	*1503*	*119*	*0.079*	***1***
Closed conformation (Sav1866 template)				
ABC-ABC contact (< 4Å)	**68**	**19**	**0.279**	**3.53** P<0.0001
ABC-ICL contact (< 4 Å)	**69**	**15**	**0.217**	**2.75** P=0.0025
Open conformation (mouse ABCB1 template)				
ABC-ICL contact (< 4 Å)	**41**	**14**	**0.341**	**4.31** P<0.0001

Table 4: Statistical analysis of the distribution of PXE-missense mutation on the domain-domain interfaces.

The mouse Abcb1 coordinates (PDB ID: 3G5U) were refined by the same group in 2013 (PDB ID: 4M1M; 3.8Å) [Li et al, 2013]. The structure improved significantly as approximately 95% of residues are in the favourable Ramachandran region compared to 57% of the original model. The registry of six transmembrane helices was corrected [Li et al, 2013]. We did not construct a new ABCC6 model according to these data yet.

Potential role of DNA-mutational hot spots in the clustering

Before we conclude, that the unequal distribution of missense mutations observed in the structure of the protein is due to the low permissiveness of distinct fundamental surfaces, we have to investigate the contribution of genetic hot spots to the observed clustering. As I have already described in the "ABCC6 sequence variation database" section, due to methylation-dependent deamination, the nucleotides of CpG sites are frequently mutated.

I determined that the frequency of CpG dinucleotides within the mutated codones of the ABC-ABC and ABC-ICL contacts were similar to that of the frequency calculated for the mutated codones (missense mutations) of the entire coding sequence (31.6%: 6 of 19 and 33.3%: 5 of 15 and 35.7%: 5 of 14 versus 31.9%: 38 of 119, respectively), indicating that most probably there is no role of CpG dinucleotides in the observed clustering of missense mutations on the domain-domain interfaces. Disease-associated missense mutations affecting ABC-ABC and ABC-ICL contact residues are represented in table 5.; substitutions that are results of CpG-site mutations are highlighted in yellow. The distribution of CpG dinucleotides within the coding sequence is represented on supp.Fig 2. Nucleotides coding for amino acid residues involved in the ABC-ABC and ABC-ICL contacts are also highlighted on this figure.

Distribution of CpG mutations on the contact surfaces	
ABC-ABC contacts closed conf.	G663C,Q698P,E699D,**G755R,R760Q,R760W**,D777N,**A781V**, L851P,G1299S,T1301I,**G1302R**,A1303P,S1307P,Q1347H, **E1400K**,S1403R,G1405S,G1501S
ABC-ICL contacts closed conf.	S398G,S398R,N497K,T500P,K502M,M751K,**R765W,R765Q**, A1139T,**R1339C,R1339H**,R1339L,I1342T,P1346S,**E1400K**
ABC-ICL contacts open conf.	S398G,S398R,N497K,T500P,K502M,E699D,**R1138W,R1138Q**, R1138P,R1273K,**R1339C,R1339H**,R1339L,**R1418Q**

Table 5: The distribution of CpG mutations on the contact surfaces of the ABC-ABC domains and that of the ABC-ICL domains in the closed confromation model as well as in the opened form. Mutations that are results of a C>T substitution within a CpG site are highlighted in yellow. All the PXE-mutations affecting the contact surfaces are listed in this table.

In summary, I have observed significant clustering of disease associated mutations on the ABC-ABC and ABC-ICL contact surfaces, that confirm the fundamental role of these domain-domain interactions in protein function predicted by Dawson et al, 2006. An inward facing conformation of Cenorhabditis elegans P-glycoprotein (PDB ID: 4F4C; 3.4Å) obtained in the absence of nucleotides and transport substrates has been also

published published in 2012 [Jin et al, 2012]. This work highlighted differences between the mouse P-gp and the C.elegans P-gp, especially in case of transmembrane helices that initiated the refinement of the previous mouse model [Jin et al, 2012; Li et al, 2013].

The predictivity of homology models

According to the limited crystal structure data of ABC exporters; there are only three "full length" structures described (PDB ID: 2ONJ 3.4Å; 3G5U 3.8A/4M1M 3.8Å; and 4F4C 3.4Å); generally we need to build models that are based on molecule structures distantly related to our protein. "Full length" indicates, that not even these three structures are complete in their length, they lack some regions, e.g. the highly flexible interconnecting loop between the two halves of the molecules. Most problematic regions during modeling are the transmembrane domains, which share less amino acid identity amongst ABC transporters than the cytosolic nucleotide binding domains. Residues of membrane helices are often replaced by similar amino acids, abundant in transmembrane regions; thus sequences of the transmembrane domains are not conserved in the ABC protein family. Even so, amino acid similarity is still high within these regions. Model building in case of large proteins, like ABC transporters, is generally thought to be reliable, when the amino acid identity of the aligned sequences is > 25-30%, see Fig.15 in the "Introduction" [Krieger et al, 2003]. We can say, in general ABC homology models are in the "safe" range, however we have to be aware of the limitations in the predictivity of our models. Homology models, based on distantly related structures, are predictive at the level of alpha carbon positions in the folding. They are unlikely to be useful in predicting details of side chain positions, or potential interactions between side chains of residues unless one focuses on highly conserved regions, e.g. like the catalytic surface of nucleotide binding folds or the ABC-ICL interacting surface. According to their crucial role in ATP hydrolysis and in intramolecular transitions they are conserved amongst ABCs; these regions may share high amino acid identity, even in case of distantly related ABCs.

On the bases of homology modelling, it is very difficult to predict the consequences of amino acid replacements in the structure, e.g. to predict the pathogenicity of a given mutation. Focusing on NBD domains, Kelly and her colleges embarked upon an analysis generating comparative models for the ABC transporters without known atomic structures [Kelly et al, 2010]. Similar to our findings in [Fulop et al, 2009], they identified conserved regions of ABC proteins based on the clustering of disease-associated mutations in several

ABC proteins. Based on the evidence, that amino acid replacements can lead to the miss function of a protein, by altering protein structure, they also attempted to train classifier programs in a supervised „random forest" learning method. Previously annotated nsSNPs were used to teach the programs to be able to distinguish between neutral and disease-associated nsSNPs. After selecting the best classifier they predicted the effect of 40, previously unclassified, nsSNP that were identified in a pharmacogenetic approach.

Actually, they did not recognize, that the majority of these sequence variations was already published previously or they were loaded to online databases. In many cases, phenotypic consequences described in patients or functional data of *in vitro* experiments were also available.

We made a thorough search in Pubmed and BioBase as well as in our ABCC6 database and we found published data on 19 of the 40 ABC nsSNPs discussed in Kellys et al [Aranyi et al, 2011]. 16 nsSNPS has been tested experimentally by others, and in 7 cases nsSNP resulted in impaired function and/or folding. 4 of these were miss-predicted by their model as being neutral changes. In 3 cases we found evidence in the literature, that the predicted neutral change in Kelly et al., is associated to a genetic disease. In 4 cases model predicted the substitution to be disease causing, while *in vitro* experiments revealed no change in protein function. In one case the predicted pathogenic change was not found to be co-segregate with disease phenotype.

In summary, in case of 10 of the 19 variant, functional consequences were mispredicted by the supervised learning method described in Kelly et al, 2010 see table 6. The data concluded here were collected and discussed in [Aranyi et al, 2011]. These results question the reliability of homology modelling used for predicting the pathogenicity of non-synonymous sequence variations in disease associated proteins, and may recommend *in vitro* experimental characterization as the preferable method.

As more and more data of genome sequencing projects are available, the question if a sequence variation is polymorphic or disease-associated becomes more and more easier to answer. However, the way that a sequence variation, that is thought to be translated, can alter protein function in most of the cases still remains unknown. Since it is impossible to characterize all the variants *in vitro* or *in vivo,* developing new *in silico* approaches predicting functional consequences of given sequence variations have great importance. Validated databases, collecting published experimental evidences as well as clinical

phenotype data of patients are extremely helpful. Either contributes to the better understanding the molecular background of a disease and protein miss-function or they can support improvements in *in silico* approaches.

Common name	HUGO name	Mutation	NBD	Predicted[1]	Published phenotype	Disease-asscociated	Protein studied *in vitro*	Reference
BSEP	ABCB11	E592Q	NBD1	NEUTRAL	Not available	—	—	—
BSEP	ABCB11	N591S	NBD1	NEUTRAL	NEUTRAL	—	Not affected	2 + 3
BSEP	ABCB11	Q558H	NBD1	NEUTRAL	Not available	—	—	—
BSEP	ABCB11	V444A	NBD1	NEUTRAL	DISEASE	Assoc. to ICP	Impaired	3
BSEP	ABCB11	E1186K	NBD2	DISEASE	Not applicable	Exon skipping	Not applicable	3 + 2
MDR1	ABCB1	P1051A	NBD2	NEUTRAL	NEUTRAL	—	Not affected	4
MDR1	ABCB1	S1141T	NBD2	NEUTRAL	DISEASE	—	Gain of function	5
MDR1	ABCB1	T1256K	NBD2	DISEASE	Not available	—	—	—
MDR1	ABCB1	V1251I	NBD2	NEUTRAL	DISEASE	—	Gain of function	6
MDR1	ABCB1	W1108R	NBD2	DISEASE	DISEASE	—	Impaired	5
MRP2	ABCC2	I670T	NBD1	DISEASE	Not available	—	—	—
MRP2	ABCC2	L849R	NBD1	DISEASE	Not available	—	—	—
MRP2	ABCC2	C1515Y	NBD2	DISEASE	Not available	Not assoc. to ICP	—	7
MRP3	ABCC3	D770N	NBD1	NEUTRAL	Not available	—	—	—
MRP3	ABCC3	K718M	NBD1	NEUTRAL	Not available	—	—	—
MRP3	ABCC3	T809M	NBD1	DISEASE	Not available	—	—	—
MRP3	ABCC3	V765L	NBD1	DISEASE	NEUTRAL	—	Not affected	8
MRP3	ABCC3	Q1365R	NBD2	DISEASE	NEUTRAL	—	Not affected	8
MRP3	ABCC2	R1297H	NBD2	DISEASE	Not available	—	—	—
MRP3	ABCC3	R1348C	NBD2	DISEASE	NEUTRAL	—	Not affected	8
MRP3	ABCC3	R1381S	NBD2	DISEASE	DISEASE	—	Impaired	8
MRP4	ABCC4	G487E	NBD1	DISEASE	DISEASE	—	Impaired	9
MRP4	ABCC4	K498E	NBD1	NEUTRAL	NEUTRAL	—	Not affect.	9
MRP4	ABCC4	R1220Q	NBD2	NEUTRAL	Not available	—	—	—
MRP4	ABCC4	T1142M	NBD2	NEUTRAL	Not available	—	—	—
MRP4	ABCC4	V1071I	NBD2	NEUTRAL	NEUTRAL	—	Not affected	9
MRP6	ABCC6	I1330L	NBD2	NEUTRAL	Not available	—	—	—
MRP6	ABCC6	I742V	NBD1	NEUTRAL	Not available	—	—	
MRP6	ABCC6	P664S	NBD1	NEUTRAL	Not available	—	—	—
MRP6	ABCC6	R724K	NBD1	NEUTRAL	NEUTRAL	Not assoc. to PXE	—	10
MRP6	ABCC6	R769K	NBD1	NEUTRAL	Not available	—	—	—
MRP6	ABCC6	A1291T	NBD2	NEUTRAL	DISEASE	Assoc. to PXE	—	11
MRP6	ABCC6	E1369K	NBD2	NEUTRAL	Not available	—	—	—
MRP6	ABCC6	G1327E	NBD2	DISEASE	Not available	—	—	—
MRP6	ABCC6	L1416R	NBD2	DISEASE	Not available	—	—	—
MRP6	ABCC6	R1268Q	NBD2	DISEASE	NEUTRAL	Homozygous not assoc. to PXE	—	12
MRP6	ABCC6	R1461H	NBD2	DISEASE	Not available	—	—	—
MXR	ABCG2	I206L	NBD1	NEUTRAL	NEUTRAL	—	Not affected	13 + 2
MXR	ABCG2	P269S	NBD1	DISEASE	NEUTRAL	—	Not affected	14
MXR	ABCG2	Q141K	NBD1	NEUTRAL	DISEASE	Yes/gout	Impaired	15 + 5

The left panel of the table (five column) is taken from Ref. 1. On the right we list our findings and their related primary reference. In several cases, we found more than one independent study showing the same result. To keep the list of references short, we indicated only one reference, if more publications were found we added $+n$ (where n is the number of additional independent publications). Neutral and disease phenotypes are shown in blue and red, respectively.
ICP, intrahepatic cholestasis of pregnancy; PXE, pseudoxanthoma elasticum.

Table 6: Summary table representing the "disease-associated" or "neutral" status of 19 variants (highlighted in blue and red), as predicted by Kelly et al, 2010 compared to that as found in the literature by Arányi et al,2011. The first 5 columns of the table represents the results of Kelly et al,2010. and are 'copy-pasted' from their publication. If a variation is predicted or published to be "disease associated", it is labelled with red, if it was predicted or published to be "neutral", it is labelled blue. Column 5. represents the predicted status of the variants by Kelly et al,2010. Status of the variants, as it was concluded by reading the published results of *in vitro* experiments or papers reporting on patients, are listed in column 6.

V.3. Functional and subcellular localization studies of disease-causing ABCC6 mutants *in vitro*

In the year 2000 genetic mutations affecting *ABCC6* locus has been identified as the genetic bases of, pseudoxanthoma elasticum (PXE) [Bergen et al, 2000; LeSaux et al, 2000; Struck et al, 2000; Ringpfeil et al, 2000]. PXE is characterized by the progressive calcification of elastic fibers in dermal, ocular and cardiovascular tissues. The transporter encoded by the *ABCC6* gene is prominently expressed in hepatocytes and to a less amount in the kidney. Neither the physiological substrate of the protein nor the pathomechanism of the disease are known. Due to a recent research hypothesis PXE is a metabolic disease [Jiang et al, 2009; Uitto et al, 2010]; ABCC6 transporter expressed at the sinusoidal surface of hepathocytes [Scheffer et al, 2002; Pomozi et al, 2013] exports (an) unidentified substrate(s) into the blood. The Inappropriate supply of the compound(s), due to missing protein activity in patients, leads to the development of calcification in peripheral tissues.

Since 2000, when the disease gene was identified more than 400 sequence variations affecting this locus were described. It was reported in 2012 that mutations in *ABCC6* gene are also responsible for a subset of GACI (Generalized arterial calcification of infancy) cases [Nitschke et al, 2012; Li et al, 2013]. GACI patients present calcification phenotype that is very similar to the characteristics of PXE, although the disease is more severe. The prenatal calcification of arterial blood vessels may cause death in early childhood. Despite the large number of 'mutations' affecting ABCC6 protein, no clear genotype-phenotype correlation in PXE has emerged. The mutation spectra of ABCC6 in PXE and the more severe disease, GACI overlap.

Mutations resulting in amino acid replacements can be extremely helpful in the better understanding of protein structure-function relations. Theoretically there are two possibilities, how amino acid substitutions can lead to the loss of function of the transporter 1) mutation affects the catalytic site of the protein, thus leads to loss of ATPase or transport activity; 2) mutation does not abolish catalytic activity but alters protein stability/folding, thus the transporter is not present in the plasma membrane in a sufficient amount. In fact, combinations of these two are also possible.

A large collaborative work started between our laboratory and research groups at the University of Hawaii, Honolulu and at Thomas Jefferson University, Philadelphia in 2009. The aim of this project is to characterize PXE-associated missense mutants of ABCC6 in

order to initiate individualized, allele specific therapeutic applications in PXE and GACI diseases. The main objective is to identify disease-associated missense mutations of ABCC6 that are transport competent, thus they are proper candidates for ”folding corrections”: to adjust their intracellular folding and trafficking by chemical chaperon molecules.

Although identification of inactive missense mutants may seem to have less significance in this project they are very important indeed. Amino acid substitutions that result in loss of transport activity may highlight fundamental residues of the catalytic and transport function.

Our laboratory contributes to this study with the analysis of ABCC6 variants within the following experimental model systems:

In vitro transport experiments with Sf9 cell derived membrane vesicles expressing ABCC6 variants are performed to identify transport competent/inactive mutants. MDCKII (Madin-Darby canine kidney) mammalian cells are used to investigate ABCC6 variants in primer subcellular localization studies *in vitro*. We have also set up a system in our laboratory capable of delivering human ABCC6 vector constructs into the liver of living mice. Abcc6 -/- mice expressing human ABCC6 variants mimic the individual *in vivo* subcellular conditions of PXE patients. Expression and subcellular targeting of ABCC6 variants as well as the effect of chemical chaperon molecules, administered *in vivo*, on protein folding can be investigated in this system. We use a chemical chaperon molecule, 4-PBA (sodium 4-phenylbutyrate) that has been proved to correct miss-folded ABC proteins in previous *in vitro* experiments published by others; ABCA1: [Sorrenson et al, 2012]; ABCA3: [Cheong et al,2006]; BSEP11/ABCB11: [Hayashi and Sugiyama, 2007 and 2009] [Lam et al, 2207]; ABCC7/CFTR: [Rubenstein and Zeitlin, 2000]. 4-PBA has significant advantages: it is a compound approved by US Food and Drug Administration (FDA) for clinical use; it does not interact with proteins directly, i.e. it does not interfere with their function [Ulloa-Aguirre et al, 2011].

For greater robustness of *in vivo* mice data parallel experiments are performed in Hawaii and Budapest.

In vivo functional rescue experiments of the developmental phenotype of morpholino-silenced zebrafish by *in vitro* transcribed mRNA of ABCC6 wt and variants were performed at Thomas Jefferson University, Philadelphia.

Our recent results were published in [LeSaux et al, 2011; Pomozi et al, 2013].

I have contributed to this project with the construction and functional analysis of disease-associated missense mutants: S1121W, T1301I, Q1347H, R1459C *in vitro*. Particularly, I have investigated their transport activity on Sf9 cell derived membrane vesicles and their subcellular localization in MDCKII (Madin-Darby canine kidney) mammalian cell line *in vitro*. I have also constructed two truncated mutants of ABCC6 that served as the negative controls in *in vivo* experiments, the most frequent PXE-mutation R1141X and the N-terminally truncated "artificial" ABCC6 mutant, Δ(2-275)ABCC6.

Construction of S1121W, T1301I, Q1347H, R1459C, R1141X and Δ(2-275)ABCC6

S1121W, T1301I, Q1347H and R1459C were selected for structure-function analysis, because they affect fundamental residues of ATP-hydrolysis or other highly conserved residues in ABCC6 protein, see Fig.26.

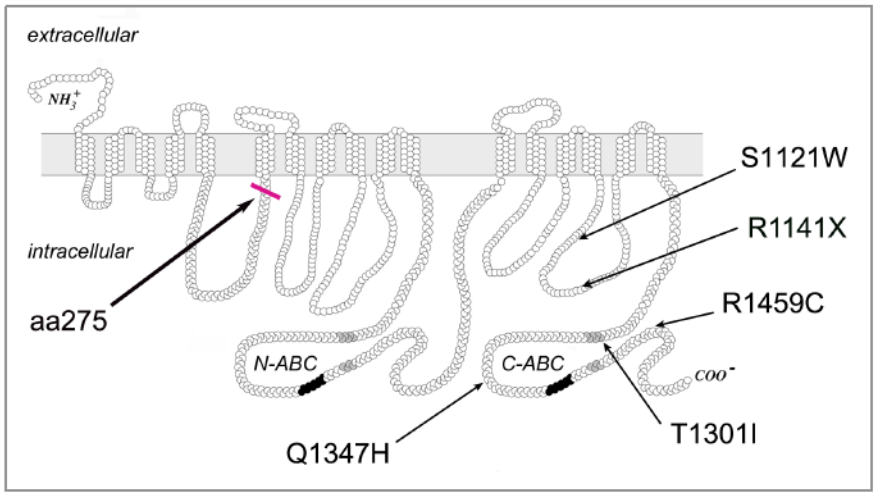

Fig.26: Position of the S1121W, R1141X, T1301I, Q1347H, R1459C amino acid replacements and the position of 275aa, the end point of the N-terminal deletion in Δ(2-275)ABCC6, in the membrane topology model of ABCC6. Conserved Walker-A and Walker-B motifs of the nucleotid binding domains are indicated in grey, while the characteristic signature motif of ABC transproters is indicated in black.

The p.S1121W mutation (c.3362C>G) [LeSaux et al, 2001] affects intracellular loop 8 (ICL8). Intracellular loops extend the helical secondary structure beyond the lipid bilayer of the cell membrane and protrude into the cytoplasm; they are thought to transmit conformational changes generated during ATP-hydrolysis in the nucleotide binding domains to the transmembrane substrate translocation pathway [Dawson et al, 2006]. This

serine residue is conserved in ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, in the mouse Abcc6, and in the zebrafish Abcca, Abcc6b and Abcc6c proteins. Protein alignment was constructed with Clustalw2 (default settings); and it is attached to the thesis as supplementary material (see supp.Fig.3, mutation is highlighted in red).

The p.T1301I mutation (c.3902C>T) [LeSaux et al, 2001] affects the Walker-A motif of the C-terminal nucleotide binding domain (C-ABC). Similar to the S1121W mutation it is conserved in ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, in the mouse Abcc6, and in the zebrafish Abcca, Abcc6b and Abcc6c proteins, for the alignment see supplementary material (see supp.Fig.3, mutation is highlighted in red).

The p.Q1347H mutation (c.4041G>C) [LeSaux et al, 2001] affects the C-terminal Q-loop in C-ABC domain. This glutamine residue is thought to have an organizing role in the coordination of the attacking water molecule in ATP hydrolysis [Jones et al, 2012]. Glutamine residue corresponding to Q1347 of ABCC6 is conserved in the majority of ABC transporters. The c.4041G>C mutation may also result in aberrant splicing since it affects the last nucleotide of exon 28. The corresponding mutation, p.Q1291H (c.3873G>C), at the last nucleotide of exon 20, in the *CFTR (ABCC7)* gene was demonstrated to cause aberrant splicing. Both correct and aberrant splice variants has been detected [Jones et al, 1992]. No proof of the splicing aberration due to the c.4041G>C mutation of ABCC6 has been published so far. The p.Q1382R amino acid substitution of the corresponding position in ABCC2 protein, see supp.Fig3, is associated to Dubin Johnson disease [Hashimoto et al, 2002].

The p.R1459C mutation [Hu et al, 2003] affects the C-terminal flanking region of the protein. The arginine residue corresponding to R1459 of ABCC6 is conserved in ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, in the mouse Abcc6 and in the zebrafish Abcca, Abcc6b and Abcc6c proteins; it is part of a conserved 'AHRL' sequence amongst these proteins, for the alignment see supp.Fig.3; mutation is highlighted in red. We have no additional information about the role of this region in protein function or trafficking.

S1121W, T1301I, Q1347H and R1459C mutants were generated by overlap extension mutagenesis PCR, as described in the "Materials and methods". Mutations were confirmed by sequencing and 'mutagenesis cassettes' were subcloned into pAcUw21L Baculovirus vector for Sf9 cell co-transfection, and into pSpSlds vector for retroviral expression in MDCKII cell line.

Two truncated mutants of ABCC6: the N-terminally truncated ABCC6 mutant, Δ(2-275)ABCC6 and the most frequent PXE mutation R1141X served as the negative controls in *in vivo* experiments. Δ(2-275)ABCC6 lacks the TMD0 and L0 domains that are characteristic features of the long type ABCC proteins (long MRPs), for an illustration see Fig.1 in the "Introduction". This mutant was originally constructed as part of a detailed analysis on the functional and structural role of N-terminal domains of ABCC6. This study is under progress, data are not described in my thesis. R1141X mutant lacks the C-terminal nucleotide binding domain of the protein, see Fig.26. This mutant was used in *in vivo* zebrafish experiments by colaborative partners at Thomas Jefferson University, Philadelphia.

R1141X and Δ(2-275)ABCC6 mutants were generated by QuikChange Site-Directed mutagenesis, as described in the "Materials and methods", and subcloned into pAcUw21-L, Bluescript II SK+ and pLIVE vectors for further studies in Sf9 cells, in zebrafish and in mice respectively.

Functional analysis of the S1121W, T1301I, Q1347H, R1459C and Δ (2-275)ABCC6 variants

For *in vitro* functional experiments Sf9 insect cells were transfected with recombinant baculovirus vector containing ABCC6 varinants: S1121W, T1301I, Q1347H, R1459C and Δ (2-278)ABCC6. Sf9 cells were cultured and membrane vesicles were prepared as described in the "Materials and methods" part.

Transport experiments were performed on 'inside out membrane vesicles' using rapid filtration assay. Here, nucleotide-binding domains face the outer surface of 'inside out membrane vesicles'. In the presence of ATP, the transporter accumulates the substrate inside the sealed vesicle. The relative amount of 'inside-out vesicles' in each sample, i. e. the transport competent vesicles, was estimated and compared to each other by measuring the ATP dependent $^{45}Ca^{2+}$ uptake of endogenous Ca^{2+} transporters. ATP dependent uptake of the radioactively labelled test substrate, LTC4 was calculated from radioactivity values measured in the presence and absence of 4mM MgATP. Transport rates were also corrected by the relative expression level of the variants as determined by immunoblot.

The transport rate of the wild type ABCC6 protein for LTC4 test substrate is very low, generally our system has the limitation to discriminate between active and inactive (activity less that 20-30%) variants of the protein. In case of the four PXE associated

variants, S1121W, T1301I, Q1347H, R1459C, transport activity was comparable to that of the wild type protein. S1121W, T1301I, Q1347H, R1459C missense mutants were concluded as transport competent mutants. LTC4 transport activity % of ABCC6 variants, as compared to that of the wild type protein, is represented on Fig.27. Fig.27 was taken from [Pomozi, Brampton, Fülöp, Chen, Apana, Li, Uitto, LeSaux and Váradi, 2014].

A residual 20-30% activity of the N-terminally truncated form of ABCC6, Δ(2-275)ABCC6 was detected. This finding is similar to that was determined for Δ(3-280)ABCC1, a close homolog of ABCC6, with amino acid identity 45%, by Eva Bakos [Bakos et al, 1998]. In these studies ΔABCC1 retained 10 % activity in Sf9 cells; the lack of the N-terminal TMD0 and L0 domains did not result in the complete loss of the protein activity, however it was rather inactive.

Functional analysis of the R1141X variant, since it lacks the C-terminal nucleotide binding domain, was not performed. The minimal requirements of a functional ABC transporter are two transmembrane and two ABC domains.

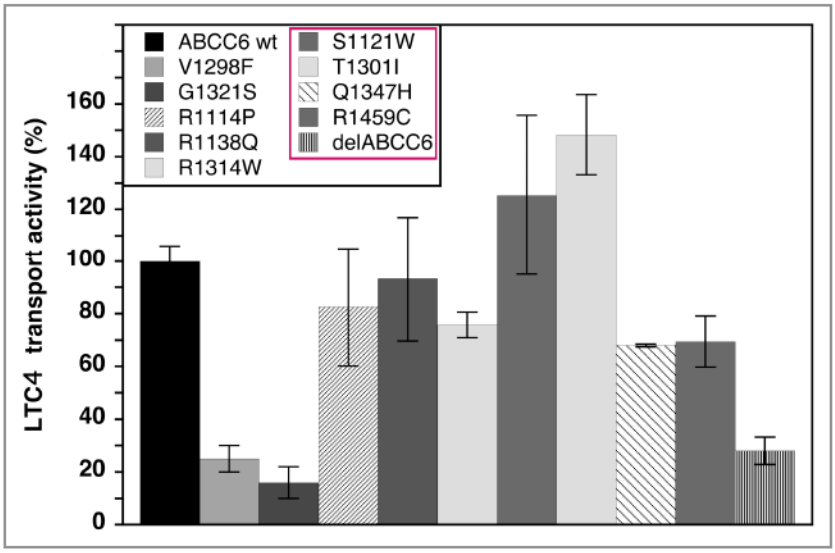

Fig.27: Transport activity values of PXE-associated missense ABCC6 variants. Figure is taken from [Pomozi, Brampton, Fülöp, Chen, Apana, Li, Uitto, LeSaux and Váradi, 2014]. ABCC6 variants shown in a red frame were constructed and investigated by me. Transport activity value of a negative control, ß-galactosidase expressing membrane, was substracted from the present transport rates of ABCC6 wt and the variants. Transport activity was detected after 0,5 min of incubation at 37 °C. Data represents the results of transport experiments performed on vesicles originating from at least two independent membrane preparations.

Functional measurements revealed S1121W, T1301I, Q1347H, R1459C missense mutants as transport active, thus they were selected for further subcellular localization analysis in MDCKII cells *in vitro*.

In vitro expression and subcellular localization studies of S1121W, T1301I, Q1347H and R1459C variants of ABCC6 protein in MDCKII cells

Using retroviral transduction, described in the "Materials and methods", I have expressed S1121W, T1301I, Q1347H and R1459C mutants in MDCKII cells. I have detected all variants with similar expression level and molecular weight as the wild type ABCC6 protein, see immunoblot on Fig.28. ABCC6 was detected by M6II-7 monoclonal antibody. ABCC6 in MDCKII cells appears with higher molecular weight compared to the form expressed in Sf9 cells corresponding to the glycosylated form of the protein, see Fig.28.

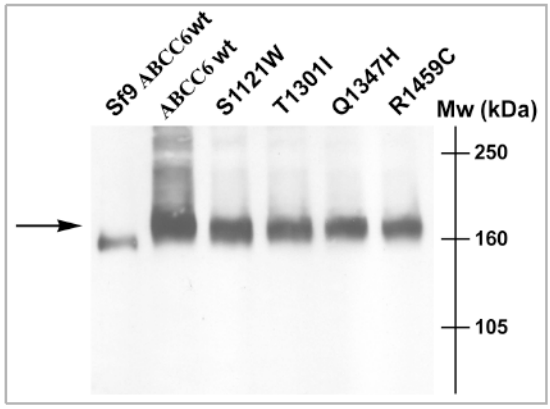

Fig.28: Immunoblot of MDCKII cell-lysates expressing ABCC6 wt and S1121W, T1301I, Q1347H, R1459C variants as developed by M6II-7 monoclonal antibody. Indicating the under-glycosylated form of the protein one sample of Sf9 cell membrane prep of the wt protein is also loaded on the gel. 30µg of cell lysates and 0.5µg of membrane preparation samples were loaded on 7% polyacryamide gel.

For primary subcellular localization studies MDCKII cells overexpressing ABCC6 wt and S1121W, T1301I, Q1347H, R1459C variants were maintained on coverslips. For fluorescent confocal imaging ABCC6 protein was labelled with M6II-7 rat monoclonal antibody and a fluorescent secondary antibody, anti rat Alexa 488. For detection Olympus IX70 microscope was used. Parallel to the experiments performed on mutant proteins, wild type ABCC6 expressing cell lines were also cultured in each experimental set up.

In my experiments ABCC6 variants showed low to high level of intracellular targeting compared to the wild type protein, however each of them could be detected in the plasmamembrane too. The intracellular targeting was more prominent in case of two mutants: T1301I and Q1347H mutants. Higher amount of the protein was localized to the plasmamembrane in case of the S1121W and R1459C. These findings were in harmony with those of Viola Pomozi, who repeated my experiments in order to investigate the effect of 4-PBA on the localization of ABCC6 missense mutants *in vitro*. I did not investigate the localization of Δ (2-275)ABCC6, these mutant was constructed prior to Viola's measurements and was included as a negative control in her study. For a representative overview of our subcellular localization experiments see fig. 29. This figure was adapted from [Pomozi, Brampton, Fülöp, Chen, Apana, Li, Uitto, LeSaux and Váradi, 2014]. ABCC6 protein is visualized in green, while the well known basolateral marker, the Na,K ATPase, in red on these figures.

In order to reach polarized monolayer, MDCKII cells were cultured on Transwell filters. Immunofluorescent labelling and confocal microscopy was performed as described previously. Parallel to the experiments performed on mutant proteins, wild type ABCC6 expressing cell lines were also cultured in each experimental set up.

Plasmamembrane localization in polarized cells was improved in case of all mutants as compared to that of detected at the nonpolarized stage. Wild type protein was properly localized to the basolateral compartment as proved by localization marker, the Na,K-ATPase, see Fig.29. S1121W and T1301I mutants showed complete plasmamembrane targeting, while Q1347H and R1459C mutants were localized to subcellular compartments too. These findings were in harmony with those of Viola Pomozi, who repeated my experiments in order to investigate the effect of 4-PBA on localization of ABCC6 missense mutants *in vitro*.

Although in most of the cases I have detected the complete plasmamembrane targeting of the variant S1121W, in some experiments besides plasmamembrane targeting I have detected significant amount of the protein intracellularly. One of these images is represented on figure 30.

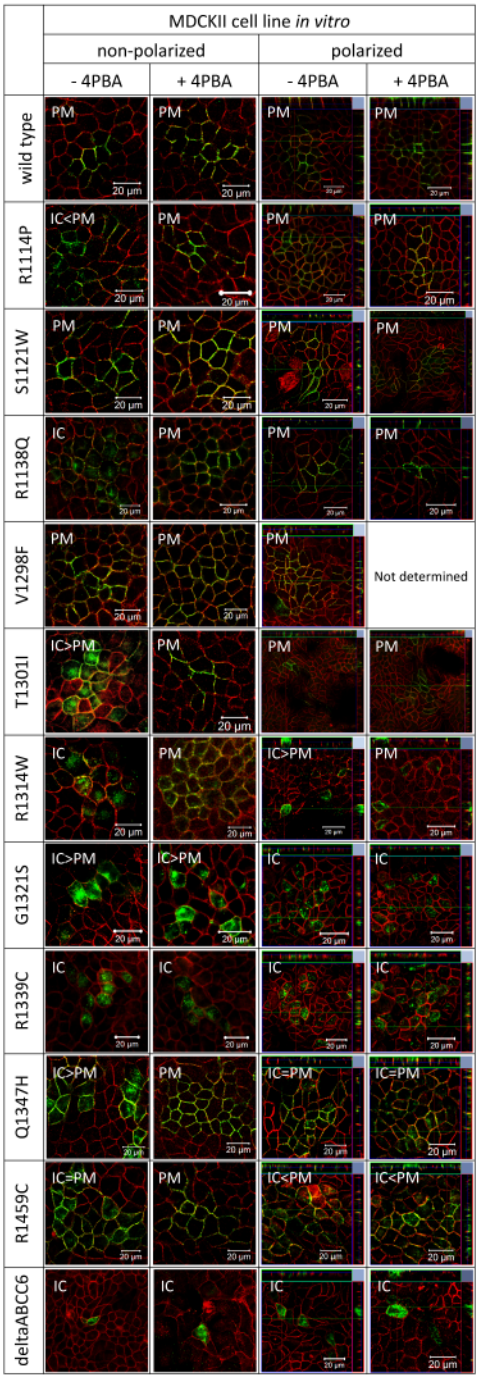

Fig.29: Subcellular localization of ABCC6 variants expressed in MDCKII cells and the effect of 1mM 4-PBA treatment on their localization. ABCC6 is detected by monoclonal antibody M6II-7 (green); the well-known basolateral marker, the Na,K-ATPase was detected with anti-Na,K-ATPase policlonal antibody (red). I have contributed to the results of untreated nonpolarized and polarized cells in case of S1121W, T1301I, Q1347Hand R1459C variants.

This finding was not included into the paper [Pomozi, Brampton, Fülöp, Chen, Apana, Li, Uitto, LeSaux and Váradi, 2014], although it further supports our conclusions that question the reliability of MDCKII cells in subcellular localization experiments of hepatic ABC transporters. In this paper we report on contradicting data of *in vitro* and *in vivo* imaging of the S1121W mutant, experiments performed by Viola Pomozi. Clear plasmamembrane targeting of the S1121W mutant was detected *in vitro* in MDCKII cells, while this mutant

showed remarkably high intracellular staining *in vivo* in mouse liver. Other experiments investigating the effect of 4-PBA on the targeting of ABCC6 mutants also revealed controversial data between the two experimental systems in Viola's hand. We have concluded that MDCKII cells have limitations, thus we propose the use of hydrodynamic tail vein injection of mice with vector constructs for transient expression *in vivo* in the liver for ABC transporters most prominent in liver.

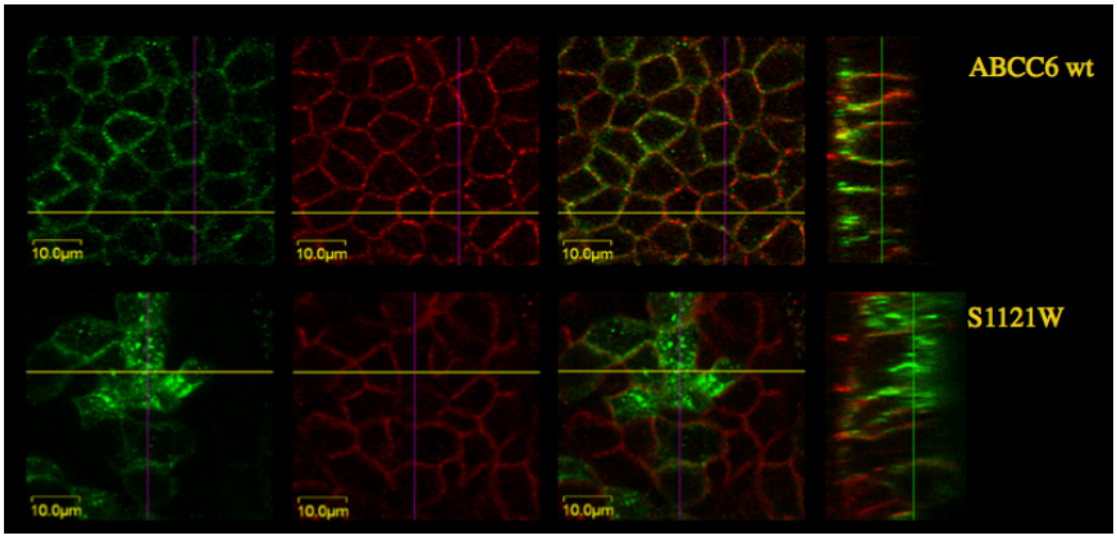

Fig.30: Intracellular localization of S1121W variant as compared to the wt protein in polarized MDCKII. A result obtained in smaller proportion amongst my experiments. ABCC6 is detected by monoclonal antibody M6II-7 (green); the well-known basolateral marker, the Na,K-ATPase was detected with anti-Na,K-ATPase policlonal antibody (red).

MDCKII cell line is widely used for recombinant protein expression. This cell line is thought to have high potential of post-translational protein processing and it is able to manage the expression of high amount of recombinant proteins, especially membrane proteins. Probably the limitations observed in our experiments are related to this phenomenon, MDCKII cells may overcome folding deficiencies of mutants with less sever instabilities.

In summary, I have expressed four PXE-associated missense mutants of ABCC6 protein; S1121W, T1301I, Q1347H, R1459C and the N-terminally truncated del(2-275)ABCC6 in Sf9 insect cells. Using LTC4 test substrate I have investigated the transport activity of the letter mutants in transport assay. My experiments revealed S1121W, T1301I, Q1347H, and R1459C to be transport competent while the N-terminally truncated

del(2-275)ABCC6 was proved rather inactive, although it preserved a marginal activity of 20-30%. S1121W, T1301I, Q1347H, R1459C ABCC6 variants showed significant intracellular targeting compared to the wild type protein in nonpolarized MDCKII cells, however they could be detected in the plasmamembrane too. The intracellular targeting was more prominent in case of the two mutants: T1301I and Q1347H mutants. Plasmamembrane localization was increased by reaching polarized status of MDCKII cells in case of all variants. S1121W and R1459C variants were localized to the plasmamembrane of polarized MDCKII in most of the cases, however, in some experiments S1121W appeared with marked intracellular trafficking. Results of nonpolarized and polarized MDCKII cell were controversial and we proposed to use *in vivo* conditions in mice over MDCKII kidney cell line.

Table 1. Summary of the characterization and rescue of disease-causing ABCC6 mutants

		Localization in mouse liver		Localization in MDCKII cell line				
				Nonpolarized		Polarized		
ABCC6 variant	Sf9 transport activity	Without treatment	After 4-PBA treatment	Without treatment	After 4-PBA treatment	Without treatment	After 4-PBA treatment	Zebrafish + mRNA rescue (%)
Wild type	Active	PM[1]	PM	PM	PM	PM	PM	90.6
R1114P	Active	IC > PM	PM (rescue)	IC < PM	PM (rescue)	PM	PM	0.0
S1121W	Active	IC > PM	PM (rescue)	PM	PM	PM	PM	7.9
R1138Q	Active	IC > PM	IC > PM (no effect)	IC	PM (rescue)	PM	PM	1.8
V1298F	<20%	PM	ND	PM	PM	PM	ND	32.0
T1301I	Active	IC > PM	IC > PM (no effect)	IC > PM	PM (rescue)	PM	PM	5.1
R1314W[1]	Active	IC > PM	PM (rescue)	IC	PM (rescue)	IC > PM	PM (rescue)	0.0
G1321S	<20%	IC	ND	IC > PM	IC > PM (no effect)	IC	IC (no effect)	0.0
R1339C	Not stable	IC	IC (no effect)	IC	IC (no effect)	IC	IC (no effect)	0.0
Q1347H	Active	IC > PM	PM (rescue)	IC > PM	PM (rescue)	IC = PM	IC = PM (no effect)	0.8
R1459C	Active	PM	ND	IC = PM	PM (rescue)	IC < PM	IC < PM (no effect)	0.0
delABCC6	<20%	IC	IC	IC	IC	IC	IC	*N.D.*
R1141X	Stop	ND	ND	ND	ND	ND	N.D.	4.8

Abbreviations: IC, intracellular; ND, not determined; PM, plasma membrane.
[1]This mutation is associated with both PXE and GACI phenotypes (Nitschke *et al.*, 2012; Li *et al.*, 2013).

Table 7: Summary of experiments targeting the functional characterization, rescue and localization studies of missense mutants in *in vitro* and *in vivo* models. Table 7. is adapted from our publication [Pomozi, Brampton, Fülöp, Chen, Apana, Li, Uitto, LeSaux and Váradi, 2014]. Cells of the table, which contain results that I have directly contributed to, are highlighted in yellow.

Further experiments *in vivo* in mouse liver by Viola Pomozi and Olivier LeSaux revealed S1121W and Q1347H missense mutants as good candidates for folding correction with chemical chaperon molecule 4-PBA. Our experiments targeting the functional characterization, rescue and localization studies of missense mutants in *in vitro* and *in vivo* models are summarized in table7 (see above). Table 7. is taken from our publication [Pomozi, Brampton, Fülöp, Chen, Apana, Li, Uitto, LeSaux and Váradi, 2014]. I have highlighted cells of the table in yellow that contain results that I have directly contributed to.

VI. DISCUSSION

This thesis concludes my contributions to the research field elucidating the physiological role of the membrane transport protein ABCC6. This protein is related to two rare genetic diseases, pseudoxanthoma elasticum (PXE; OMIM: 264800) and generalized arterial calcification of infancy (GACI; OMIM: 208000), with unknown pathogenic background. The transporter is prominently expressed in the liver, in the basolateral plasma membrane of hepatocytes [Pomozi et al, 2013], see Fig.16, and it is thought to be involved in the excretion of (an) unknown substrate(s) into the blood, which directly or indirectly prevents the calcification of elastic fibers in the lamina intima of middle sized arteries and other soft tissues [Jang et al., 2009; Uitto et al., 2010].

Rare genetic diseases may draw our attention to special pathologic malfunctions that are characteristic to disorders; they can help in revealing crucial steps of complex physiologic processes. This keeps researchers focusing on rare genetic disorders, makes them to establish and test new hypothesises in order to elucidate the molecular background of a previously unknown pathologic mechanisms. In 2007 a new disease has been described with analogous connective tissue calcification defects to PXE [Vanakker et al., 2007]. Peripheral calcification symptoms were so closely related that the name was given to the new disease: „PXE-like-syndrome". Crucial difference between the two disorders is the multiple coagulation factor deficiency that is only characteristic to „PXE-like disorder" but not to PXE. In „PXE-like disease" mutations affect the *GGCX* gene that codes for the gamma-glutamyl carboxylase enzyime, responsible for the post synthetic carboxylation of Gla-domain containing proteins, see Fig.12, conferring Ca-chelating properties for those. The GGCX enzyme needs VitK as cofactor in its functional redox-cycle.

These new findings highlighted Vitamin K forms as potential substrate-candidates of ABCC6 in PXE. According to this hypothesis ABCC6 would transports vitamin K metabolite(s) from the liver to the peripheral tissues, where these compounds are utilized during the gamma-glutamyl carboxylation of Ca-chelating factors that prevent calcification of elastic tissues [Borst et al., 2008]. PXE patients develop calcification symptoms due to deficit in VitK supply at the periphery but maintain normal level of blood-clotting factors since VitK levels are sufficient in the liver.

The exact metabolic pathway of VitK is not known currently. The majority of vitamin K is dietary uptaken in the form of VitK1, phylloquinone. Most of it is utilized in the liver and serves as cofactor in the synthesis of blood coagulating factors. Via side-chain removal VitK1 can be converted to an intermediate form, VitK3 (menadione), see Fig.13. As being a slightly cytotoxic and also a hydrophobic compound, VitK3 might be eliminated from hepatocytes via conjugation to glutathione (GSH) or glucuronide molecules. Similar conjugates are frequently eliminated from the cells by ABC proteins: ABCC1, ABCC2, ABCC3 and ABCG2 [Jedlitschky et al, 1994; Leier et al, 1994; Loe et al, 1996; Deeley et al, 2006; Oude et al, 1989 Paulusma et al, 1999; Zeng et al, 2000; Zelcer et al, 2001]. There are several evidences published in the literature that VitK2 (menaquinone), the VitK form utilized by peripheral tissues, is generated from VitK1 through the VitK3 intermedier form [Okano et al., 2008]. According to these, VitK3, and thus the conjugated water-soluble forms, could serve as sources of the peripheral VitK supply.

The first section of my thesis concludes my contribution to the research elucidating the potential role of the VitK3 metabolite, especially that of the glutathione (GSH) conjugated form (VK3GS), in the pathologic background of ABCC6 associated diseases.

VI.1. Testing VK3GS as substrate candidate for ABCC6 ABCC1, ABCC2, ABCC3 and ABCG2 hepatic transporters *in vitro*

ABCC6 was previously demonstrated to be able to transport glutathione conjugates [Iliás et al., 2002], also see Fig.11, thus I focused on the question whether ABCC6 is able to transport VK3GS, the glutathione conjugated form of the VitK3 metabolite.

Since this is a slightly cytotoxic, hydrophobic compound with relevant physiological function four additional hepatic ABC transporters, ABCC1, ABCC2, ABCC3 and ABCG2, previously demonstrated to be able to transport glutathione conjugated metabolites [Jedlitschky et al, 1994; Leier et al, 1994; Loe et al, 1996; Deeley et al, 2006; Oude et al, 1989 Paulusma et al, 1999; Zeng et al, 2000; Zelcer et al, 2001], were also involved in the study.

I tested all the above ABC transporters *in vitro* in vesicular transport measurements performed on Sf9 insect vesicles. For these experiments I have synthesized and purified unlabeled and radioactively labelled VK3GS conjugate from VitK3 and GSH or [^{3}H]GSH, respectively. I have investigated the ATP-dependent VK3GS transport of ABCC6, ABCC1, ABCC2, ABCC3 and ABCG2 transporters on validated membrane vesicles.

In case of ABCC6 transporter I could not detect significant level of VK3GS-transport, see Fig.18, indicating that ABCC6 is most probably not involved in the export of VK3GS from hepathocytes [Fülöp et al., 2011].

In similar experimental conditions ABCC1 transporter was proved to be a high capacity transporter of VK3GS, with approximate K_M and V_{max} values of 1.45μM and 240 pmol/mg membrane protein/min, respectively [Fülöp et al., 2011], also see Fig.18 and 21. This transport was specific, as 500nM of LTC4, the physiological substrate of ABCC1 [Leier et al, 1994; Jedlitschky et al, 1994; Loe et al, 1995], and the common MRP-inhibitor, MK571 (10μM) inhibited the transport to a residual activity of 35% and 23%, respectively, see Fig.22. No ATP dependent transport of the VK3GS compound was observed in case of the ABCC1 inactive mutant G771D, see Fig.18.

These assays also revealed ABCC2 as a potential low capacity transporter of the VK3GS conjugate, see Fig.18 and 20. No transport of VK3GS by ABCC3 and ABCG2 transporters could be detected amongst similar conditions in our transport system, see Fig.18.

My results reporting on ABCC6 and ABCC1 transporters were in harmony with *in vitro* vectorial transport experiments performed by our collaborative partners in the Netherlands Cancer Institute, Amsterdam. No significant difference compared to control MDCKII cell was observed in case of ABCC6 overexpressing cells, while ABCC1 overexpressing cells showed a remarkable increase in apical–to-basal efflux relative to control cells [Fülöp et al., 2011]. *In vivo* liver perfusion experiments of wild type and *Abcc6-/-* mice, performed parallel in our laboratory by Viola Pomozi and by collaborative partners in the Thomas Jefferson University, Philadelphia, also supported our findings. VitK3 was administered into the portal vein of mice. The glutathione-conjugated form of VitK3 (VK3GS) in the outflow fluid of the inferior vena cava was detected only when VitK3 was added before (HPLC-MS assay of VK3GS was performed by Pál Szabó, MTA KKI.). No significant difference in the VK3GS levels of the outflow fluid was detected between wild type and *Abcc6-/-* mice [Fülöp et al., 2011].

All these above findings argue against the role of ABCC6 in the elimination of VK3GS metabolite from hepathocytes and were published in [Fülöp et al., 2011].

The second and third sections form a cohesive part of my thesis. Both of them focus on disease-associated mutations of the ABCC6 transporter. The second section describes the

ABCC6 mutational database that is a validated collection of ABCC6 mutations reported in the literature of PXE and GACI diseases, both related to mutations in *ABCC6* gene. This part describes the generation of the ABCC6 homology model too [Fulop et al., 2009]. The distribution of PXE associated missense ABCC6 mutations in the predicted protein structure was also studied [Fulop et al., 2009], and my results are concluded in this part.

VI.2.1. ABCC6 sequence variation database:

ABCC6 database was first established in 2007 in our laboratory by Orsolya Symmons and me, see supp.tables1-7. This database lists all the PXE and GACI related ABCC6 mutations that has been published in the literature. Most frequently used information connected to each sequence variation/entry are listed in columns "A-I" of the table. These data are as follows: the sequence variation at DNA level; the sequence variation at protein level; the status: mutation/polymorphism; the type of alteration; the genomic position of the alteration; the genomic region affected; the protein region affected; in case of a "C>T" variation if it has occurred at a "5'CpG". All publications that report on a particular sequence variation are given. In every case where it was available allele frequency data is also indicated.

Overall in the *ABCC6 gene* we have collected 220 disease causing mutations (supp.tab.2.) and 154 variations (supp.tab.3.) that are described as polymorphisms in the literature. Besides the large number of intronic single nucleotide substitutions, we found 10 missense amino acid alterations and 22 silent amino acid substitutions that are reported as polymorphisms (supp.tab.6.). The 220 PXE associated mutations are as follows: 11 intronic single nucleotide substitutions, that most probably cause miss-splicing of the mRNA; 1 small deletion spanning an exon-intron barrier; 23 large deletions; 38 small deletions, insertions or duplications, the majority of these are out of frame mutations (supp.tab.4.); and 147 single nucleotide substitutions, that result in 124 missense and 23 nonsense alterations (supp.tab.5.). The missense and nonsense amino acid substitutions contribute 67 % to the total number of PXE causing mutations.

I have also analyzed the CG content of the 4512 nucleotide long cDNA as well as the distribution of CpG dinucleotides within the coding region, see supp.Fig.2. GC content of the cDNA is 60.6%, while the total number of CpG dinucleotides is 156. In our collection of ABCC6 sequence variations we have found 205 single nucleotide substitutions that affect the coding region; 59 (29%) of these occurred on CpG sites, see supp. table 7.

We concluded that the distribution of single nucleotide substitutions within the coding sequence is not random, CpGs are more frequently affected. 29% of the single nucleotide substitutions affecting the coding region occurred on 312 bases (156 5'CpG sites) that correspond to the 6,9 % of the coding nucleotides. Similar as it was reported in case of several genetic diseases [Holliday et al, 1993] mutational events affecting CpG dinucleotides largely contribute to the number of disease causing mutations. Mutations identified on CpG sites along the *ABCC6* gene (45, see supp.table 7.) represent the 20.5 % of all disease causing mutations (220), based on the data reported until 2013. The most frequent disease causing mutation, found in 20-30 % of the PXE alleles [Pfendner et al, 2008], the c.3421C>T; p.R1141X, is also located to a CpG site, as Arg1141 is encoded by the CGA triplet.

In 2008 PXE international, the American advocacy organisation of PXE patients (www.pxe.org), initiated collaboration in order to synthesize our data sets and establish an online open database for ABCC6 sequence variations [Váradi et al., 2011]. Since June 13, 2013 ABCC6 database operates on the server of the NCBI powered by an LOVD platform: `http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6` (also see supp.Fig.1.). The collection of ABCC6 variants, generated in Budapest by Orsolya Symmons and me, served as the major basis in establishing the online database. Clinical data according to Phenodex index, a validated registry of the phenotypic stages of symptoms described amongst PXE patients, were loaded into the database by PXE International. Despite the large number of PXE-associated ABCC6 mutations, published thus far, no clear genotype-phenotype correlation has emerged.

VI.2.2. ABCC6 homology model:

The homology model of ABCC6 [Fulop et al, 2009], also linked to the online database, gives an opportunity to answer structural and/or functional consequences of mutations via embedding missense mutations in a structural view of the protein. One can easily visualize the localization of the ABCC6 variants in the three-dimensional model of the protein. It is an interactive surface of the database and hence it is a very useful tool in initiating or testing new research ideas related to mutations described in the protein structure.

This model that we have generated on the basis of the coordinates of the Sav1866 transporter [Dawson RJ et al, 2006 and 2007] in 2009 was the first homology model of ABCC6 protein [Fulop et al., 2009], also see Fig.23. The coordinates of ABCC6 homology

model are available at http://www.enzim.hu/~varadi/ABCC6/. In March 2009 the crystal coordinates of mouse Abcb1 (PDB ID: 3G5U), in the nucleotide-free inward-facing conformation, were published [Aller et al., 2009]. We have repeated model building using the above, inward-facing structure coordinates, see Fig.23 [Váradi et al, 2011].

We have investigated the distribution of PXE-associated missense mutationos on functional domain-domain interfaces of the protein and found significant clustering of them on the ABC-ABC interface and on the ABC-ICL contact surface [Fulop et al., 2009].

Amino acid residues that are predicted to be situated within 4Å distance to residues of the opposite ABC domain or to the MgATP nucleotide in the *outward-facing conformation* of ABCC6 were investigated first. According to the model 68 amino acid residues were found to be potentially involved in the above interaction, and out of these 19 are affected by mutations described in PXE patients, see table3. This ratio (19/68) is 3.53 fold higher than the overall mutational frequency (referring to missense mutations) of the molecule (119/1503) ($p<0.0001$), indicating that this part of the protein structure is significantly less permissive for missense mutation, see table 4.

69 amino acid residues in the *outward-facing ABCC6 model* that meet the 4Å criteria were selected to be potentially involved as forming the transmission interface of ABC domains and the four coupling helices of the TMD1 and TMD2 domains. 15 of these were affected by PXE mutations, see table3. The mutational rate of this area (15/69) is 2.75-fold higher than that of the whole molecule (119/1503)($p=0.0025$), see table4. The high frequency of disease causing mutations suggests, that the ABC-ICL interacting surface is less tolerant to missense substitutions, than the overall ABCC6 transporter. This is in harmony with the finding of Dawson et al., 2006 about the Sav 1866 structure, predicting a fundamental role for these domain-domain surfaces in ABC protein function.

Since NBDs are oriented far from each other in the *inward-facing structure*, the distribution of mutations could be investigated only in case of the ABC-ICL surface. Similar to our previous findings a 4.31 fold accumulation of disease-associated mutations was detected on this contact surface, see table4. 14 of the 41 amino acid residues that meet the 4Å criteria are affected by PXE mutations, see table3.

I have also investigated the potential contribution of 5'CpGs as genetic hot spots to the observed clustering of missense disease-associated mutations on the ABC-ABC and ABC-ICL contact surfaces. I determined that the frequency of CpG dinucleotides within the

mutated codones of the ABC-ABC and ABC-ICL contacts were similar to that of the frequency calculated for the mutated codones (missense mutations) of the entire coding sequence (31.6%: 6 of 19 and 33.3%: 5 of 15 and 35.7%: 5 of 14 versus 31.9%: 38 of 119, respectively), indicating that most probably there is no role of CpG dinucleotides in the observed clustering of missense mutations on the domain-domain interfaces.

In summary, I have observed significant clustering of disease associated mutations on the ABC-ABC and ABC-ICL contact surfaces. These findings were the first genetic proof supporting the fundamental role of the ABC-ICL intramolecular surfaces, first highlighted during the analysis of the Sav1866 structure in [Dawson et al., 2006].

Based on homology modeling, it is very difficult to predict the consequences of a given amino acid replacement in the protein structure, e.g. to predict the pathogenicity of an observed sequence variation. Even so it might be successful if someone focuses on conserved regions of a protein, like the NBD domains of ABCs.

Kelly and her colleges embarked upon an analysis generating comparative models for the NBDs of ABC transporters without known atomic structures [Kelly et al, 2010]. Similar to our work [Fulop et al, 2009], based on the clustering of disease-associated mutations, they identified conserved regions in several ABC proteins. Using previously annotated nsSNPs they trained classifier programs in a supervised „random forest" learning method to be able to distinguish between neutral and disease-associated nsSNPs. After selecting the best classifier they predicted the effect of 40 previously unclassified nsSNP identified in a pharmacogenetic approach.

Actually, they did not recognize, that the majority of these sequence variations was already published previously, or they were loaded to online databases. In many cases, phenotypic consequences described in patients or functional data of *in vitro* experiments were also available. We made a thorough search in Pubmed and BioBase as well as in our ABCC6 database and we found published data on 19 of the 40 ABC nsSNPs discussed in Kellys et al [Arányi et al, 2011]. In summary, in case of 10 of the 19 variant, functional consequences were mispredicted by the supervised learning method described in Kelly et al, 2010, also see table 6. The data concluded here were collected and discussed in [Arányi et al, 2011].

However these results might question the usefulness of homology modelling in predicting the pathogenicity of non-synonymous sequence variations it is not the case in general.

Since it is impossible to characterize all the protein variants described in genome sequencing projects using regular *in vitro* or *in vivo* methods, the development of appropriate *in silico* approaches predicting functional consequences of sequence variations have great importance.

In vitro cell free activity assays as well as *in vitro* and *in vivo* cellular models expressing recombinant proteins are frequently used model systems investigating disease-associated variants of a protein. These are conventional approaches that may lead to the better understanding of the molecular mechanisms of a protein. In the third section of my thesis I conclude my experiments investigating structural and functional consequences of missense ABCC6 mutations in *in vitro* transport and *in vitro* subcellular localization experiments. In this section I also conclude our contribution to a collaborative study initiating the first mutation based therapeutic application in PXE and GACI diseases [Pomozi, Brampton, Fülöp, Chen, Apana, Li, Uitto, LeSaux and Váradi, 2014].

VI.3. Functional and subcellular localization studies of disease-causing ABCC6 mutants *in vitro*:

We have embarked upon a large research project investigating missense mutations of ABCC6 protein, related to GACI or PXE diseases, in order to elucidate the molecular mechanisms of the transporter. In a collaborative work with research groups at the University of Hawaii, Honolulu and at Thomas Jefferson University, Philadelphia ABCC6 protein variants are characterized in *in vitro* and *in vivo* experimental systems. Theoretically, there are two possibilities, how amino acid substitutions can lead to the loss of function of the transporter 1) mutation affects the catalytic site of the protein, thus leads to loss of ATPase or transport activity; 2) mutation does not abolish catalytic activity but alters protein stability/folding, thus the transporter is not present in the plasma membrane in a sufficient amount. In fact, combinations of these two are also possible.

The main objective of our study is to identify disease-associated missense mutations of ABCC6 that are transport competent, thus they are proper candidates for ”folding corrections”: to adjust their intracellular folding and trafficking by chemical chaperon molecules. Successful *in vitro* and *in vivo* “folding correction” experiments may serve as first steps toward allele specific therapeutic applications in PXE and GACI diseases. We use a chemical chaperon molecule, 4-PBA (sodium 4-phenylbutyrate) that has been proved to correct miss-folded ABC proteins in previous *in vitro* experiments published by others;

ABCA1: [Sorrenson et al., 2012]; ABCA3: [Cheong et al.,2006]; BSEP11/ABCB11: [Hayashi and Sugiyama, 2007,2009] [Lam et al., 2207]; ABCC7/CFTR: [Rubenstein and Zeitlin, 2000]. 4-PBA has significant advantages: it is a compound approved by US Food and Drug Administration (FDA) for clinical use; it does not interact with proteins directly, i.e. it does not interfere with their function [Ulloa-Aguirre et al, 2011].

In our laboratory we have developed an *in vitro* experimental systems to be able to discriminate between transport competent and inactive mutants of ABCC6 protein. ABCC6 variants are tested in functional measurements performed on Sf9 insect cell derived vesicles. We use LTC4 test substrate that was previously proved to be transported by the wild type protein [Iliás et al., 2002], see Fig.11.

I have contributed to this project with the construction and functional analysis of the following disease-associated missense mutants: S1121W, T1301I, Q1347H, R1459C, see Fig.26, *in vitro*. Particularly, I have investigated their transport activity on Sf9 cell derived membrane vesicles and found that all the four PXE associated variants, S1121W, T1301I, Q1347H, R1459C have transport activity that is comparable to that of the wild type protein, see Fig.27. I have also constructed two truncated mutants of ABCC6 that served as the negative controls in *in vivo* experiments, the most frequent PXE-mutation R1141X and the N-terminally truncated "artificial" ABCC6 mutant, Δ(2-275)ABCC6, see Fig26. A residual 20-30% activity of the N-terminally truncated form of ABCC6, Δ(2-275)ABCC6 was detected in *in vitro* LTC4-transport experiments, see Fig.27. This finding is similar to that was determined for Δ(3-280)ABCC1, a close homolog of ABCC6, with amino acid identity 45%, by Eva Bakos [Bakos et al., 1998]. Functional analysis of the R1141X variant, since it lacks the C-terminal nucleotide binding domain, was not performed.

As S1121W, T1301I, Q1347H, R1459C missense mutants were concluded as transport competent mutants I started to investigate their subcellular localization.

I performed *in vitro* experiments on MDCKII (Madin-Darby canine kidney) mammalian cell line. I have expressed these four variants in MDCKII cells using retroviral transduction. I have detected all variants with similar expression level and molecular weight as the wild type ABCC6 protein when they were investigated on immunoblot, see Fig.28. For primary subcellular localization studies MDCKII cells overexpressing ABCC6 wt and S1121W, T1301I, Q1347H, R1459C variants were maintained on coverslips. I have detected low to high level of intracellular targeting of ABCC6 variants compared to the

wild type protein, however each of them could be detected in the plasmamembrane too. The intracellular accumulation was more prominent in case of two mutants: T1301I and Q1347H. Higher amount of the protein was localized to the plasmamembrane in case of the S1121W and R1459C, for representative images published in Pomozi, Brampton, Fülöp, Chen, Apana, Li, Uitto, LeSaux and Váradi, 2014 see Fig.29. These findings were in harmony with those of Viola Pomozi, who repeated my experiments in order to investigate the effect of 4-PBA on the localization of ABCC6 missense mutants *in vitro and in vivo*. I did not investigate the localization of Δ(2-275)ABCC6, these mutant was constructed prior to Viola's measurements and was included as a negative control in her study.

In order to analyze the subcellular localization of the variants in polarized cells that is a characteristic feature to hepathocytes and the tubular epithelial cells of the kidney, MDCKII cells were cultured on Transwell filters. I have detected the improved plasmamembrane localization of all mutants in polarized monolayer as compared to that of detected at the nonpolarized stage. S1121W and T1301I mutants showed complete plasmamembrane targeting, while Q1347H and R1459C mutants were localized to subcellular compartments too, for representative images published in Pomozi, Brampton, Fülöp, Chen, Apana, Li, Uitto, LeSaux and Váradi, 2014 see Fig.29. These findings were in harmony with the later findings of Viola Pomozi, who repeated my experiments in order to investigate the effect of 4-PBA. Although in most of the cases I have detected the complete plasmamembrane targeting of the variant S1121W, in some experiments I also detected significant intracellular targeting, see Fig.30.

This finding was not included into the paper [Pomozi, Brampton, Fülöp, Chen, Apana, Li, Uitto, LeSaux and Váradi, 2014], although it further supports our conclusions that question the reliability of MDCKII cells in subcellular localization experiments of hepatic ABC transporters. Based upon these results we propose the use of hydrodynamic tail vein injection of mice with vector constructs for transient expression *in vivo* in the liver for ABC transporters most prominent in liver.

MDCKII cell line is widely used for recombinant protein expression. This cell line is thought to have high potential of post-translational protein processing and it is able to manage the expression of high amount of recombinant proteins, especially membrane proteins. Probably the limitations observed in our experiments are related to this

phenomenon, MDCKII cells may overcome folding deficiencies of mutants with less sever instabilities.

In this paper we reported on the characterization of ten frequent disease-causing missense mutations of ABCC6 (R1114P, S1121W, R1138Q, V1298F, T1301I, R1314W, G1321S, R1339C, Q1347H, R1459C). We proved seven of the ten missense variants to be transport competent in Sf9 cell derived membrane vesicles. One of the mutants, R1339C, was found to be unstable in Sf9 cells. We characterized the subcellular localization of the variants *in vitro* in MDCKII cell line and *in vivo* in mice. In nonpolarized cells only S1121W and the inactive V1298F were fully targeted to the plasmamembrane. In polarized MDCKII cells, besides S1121W and V1298F, R1114P, R1138Q and T1301I variants were also found in the basolateral plasmamembrane, see Fig.29.

After *in vitro* experiments, each mutant was individually expressed *in vivo* in mouse liver, and frozen sections have been analyzed by immunohistochemical labelling. The R1459C and the inactive V1298F mutant were shown to have identical subcellular locatlization to the wild type protein *in vivo*. All the other variants were found to various extent intracellularly.

In pharmacological rescue experiments the effect of 4-PBA on the subcellular targeting of disease associated variants has been tested in MDCKII cells as well as in the liver of living mice. These experiments resulted in controversial data between data originating from the MDCKII cell line and from liver sections of living mice, see table 7., contraindicating MDCKII cells as feasible system for subcellular characterization of liver resident proteins. 4-PBA treatment was effective in case of Q1347H expressed in nonpolarized MDCKII or *in vivo* in mice but not in polarized MDCKII. It was the same in case of R1459C mutant except the effect was not investigated *in vivo* since in this condition R1459C was fully targeted to the cell membrane without any treatment. Effect of 4-PBA in nonpolarized MDCKII was similar in case of R1138Q, T1301I however the treatment did not induced plasmamembrane rescue *in vivo* in mice. The only case where all experimental conditions resulted in similar rescue was the R1314W mutant, where 4-PBA was proved to be effective in both MDCKII systems as well as *in vivo* in mice. No plasma membrane targeting could be achieved in case of R1339C mutant irrespective of the experimental system that has been used.

The functionality of the mutants was also tested *in vivo* in phenotypic rescue experiments in zebrafish. It has been proved earlier that human *ABCC6* mRNA provides near complete rescue of the developmental phenotype induced by morpholino-mediated silencing of *ABCC6a* gene in zebrafish embryos [Li et al., 2010; Zhou et al., 2013]. Here, in [Pomozi, Brampton, Fülöp, Chen, Apana, Li, Uitto, LeSaux and Váradi, 2014] besides the full rescue of the wt human mRNA of *ABCC6* (90.6%) only V1298F, the inactive mutant, showed significant rescue, see table7. S1121W showed 7.9%, otherwise all the other mutants resulted in similar effect (0-5.1%) to that of the control mRNA, the R1141X, a nonsense mutant of ABCC6, 4,8%. 4-PBA in concentrations used in MDCKII or in mice was toxic to zebrafish, thus further experiments with this compound could not be performed in this system.

Briefly, during my PhD period I have contributed to the research elucidating the molecular background of two heritable disorders pseudoxanthoma elasticum and generalized arterial calcification of infancy by the following works:

I have investigated the physiological function of ABCC6 protein in a hypothesis based study, testing a VitK metabolite as potential substrate.

I have also contributed to the field by participating in establishing the ABCC6 sequence variation database and in the generation of homology models of ABCC6 protein. These *in silico* works may contribute to the better understanding of protein structure and function evidences and initiate new research ideas by cataloguing the numerous disease associated mutations and by providing possibility to arrange them in the structural view of the protein.

As part of a large collaborative work I have performed *in vitro* experiments on disease associated variants of ABCC6. My results contributed to a work that may serve the basis of mutation based allele specific therapy in PXE and GACI diseases.

Based on the estimated prevalence, 1 in 50.000, PXE (OMIM: 264800) is considered as a rare disease. GACI (OMIM: 208000) is an extremely rare condition; there are approximately 180 patients registered today. The general definition of “rare disease” varies worldwide. According to the EURORDIS (The Europian Organisation for Rare Diseases) “rare disease” in Europe is specified as a disease that occures infrequently in the general population; it affects less than 1 person in 2000 citizens (EC Regulation on Orphan

Medical Products). While 1 out of 2000 seems very few, according to a well-accepted estimation [WHO Report on Priority Medicines for Europe and the World, 2004; for a pdf version see: http://archives.who.int/prioritymeds/report/final18october.pdf], rare diseases affects about 30 million individuals in the 25 Europian countries involved in the study, and approximately 6 to 8 % of the total Europian population. Unfortunately, data are often inadequate since people with rare diseases are not frequently registered to accurate databases. Moreover, in certain cases rare diseases are described as "other endocrine and metabolic disorder". According to estimation 5.000 to 7.000 distinct rare diseases exist [Eurordis: Rare diseases: Understanding this Public Health Priority, 2005; for a pdf. Version see: http://www.eurordis.org/IMG/pdf/princeps_document-EN.pdf].
80% of rare diseases have identified genetic origin and they concern 3 to 4 % of births. The remaining part is caused by either infections, allergies, degenerative processes or teratogenic causes, while some are related to combined genetic and environmental factors [Eurordis: Rare diseases: Understanding this Public Health Priority, 2005; for a pdf. Version see: http://www.eurordis.org/IMG/pdf/princeps_document-EN.pdf]. In most of the cases, due to the lack of research, the exact ethiological origin still remains unknown.

Since rare disease patients represent the minority of citizens, there used to be a lack of public awareness for a long time in the past. The pharmaceutical market is so narrow that companies do not invest in the research and the development of treatments. Recognizing this, the National Organization for Rare Disorders (NORD) was founded in the 1970s. The organization serves for emphasizing the need for economic incentives in promoting drug research for "orphaned" diseases. As the resources are limited and patient populations are small there is also a need for international cooperation in scientific research. Advocacy organizations, like the PXE International, the American advocacy organization of PXE patients, may largely promote communication between patient groups and researchers worldwide. They are also effective in lobbying for governmental support as well as for other financial investments. Drugs for rare diseases, including conditions that are not intrinsically rare but there is no reasonable expectation that a treatment will be developed without governmental assistance, were referred as “orphan drugs” in the Federal low: Orphan Drug Act of 1983, in the US. It was installed in order to promote the development of drugs and medical devices by making grants as well as to coordinate among Federal agencies, manufacturers and organizations representing patients.

Developing new drugs takes enormous amount of time, efforts and money. "Drug repurposing/repositioning" or "off-label use" may provide treatment in certain orphan conditions. "Off-label use" means the prescribing of a medication by a doctor in a manner different from the originally approved practice. However off-label use became very common in the last ten years, in case of the 3 leading drugs in each of the 15 leading drug classes off-label use accounted for approximately 21% of the prescriptions in 2003 [Stafford, 2008], in most of the cases it lacks adequate supporting data. Evidence provided for one clinical condition should not be automatically applied to others [Stafford, 2008; Radley et al, 2006]. Thus FDA (Food and Drug Administration, US) prohibits the direct promotion of a product for unapproved uses [Stafford, 2008]. Researchers and manufacturers are turning to the strategy of "drug repurposing/repositioning", i.e. they are investigating small molecules already approved by FDA to treat one disease or to those that previously passed toxicity tests but were aborted in further drug development to see whether these are safe and effective in treating other diseases. By reducing time frame and costs it might significantly improve success rates in case of orphan conditions. With the *in vitro* and *in vivo* test of 4-phenylbutyrate we started a study that can be referred as drug repurposing/respositioning.

In 2012 the NIH via the National Center for Advancing Translational Sciences (NCATS) started a collaborative program coordinating NIH-founded research and pharmaceutical companies providing researchers with high-quality molecular compounds that already cleared several steps along drug development. The *NCATS Pharmaceutical Collection* was also published within the program in 2011, that is a publicly available database of 3.800 small molecules registered for clinical trials in Canada, Europe and Japan.

VII. SUMMARY

ABCC6 protein is a transmembrane transporter that is predominantly expressed in the basolateral plasmamembrane compartement of hepathocytes and epithelial cells of the proximal tubules in the kidney. Mutations in the *ABCC6* gene are the genetic basis of two Mendelian disorders, pseudoxanthoma elasticum (PXE) and generalized arterial calcification of infancy (GACI), characterized by dystrophic calcification affecting extrahepatic tissues, e.g. soft tissues within the skin, eye and middle sized arteries. PXE and GACI diseases are considered as metabolic disorders and the ABCC6 transporter is thought to transport (an) unidentified substrate(s) from the liver to the circulation that directly or indirectly prevents ectopic soft tissue calcification in organs affected by PXE and GACI diseases.

Based on *in vitro* transport experiments I disproved a recent hypothesis suggesting a Vitamin K metabolite, the VK3GS conjugate, as potential physiological substrate of the ABCC6 transporter. *In vitro* and *in vivo* data of others also supported my results, thus I concluded that ABCC6 protein is not involved in the elimination of VK3GS conjugate from hepathocytes. My *in vitro* transport experiments also revealed ABCC1 as a high capacity transporter of the VK3GS metabolite.

I have contributed to the establishment of a validated database collecting the published sequence variations of *ABCC6* gene. These data served as the major basis of the online "ABCC6 LOVD" database. I also investigated the contribution of "5'CpGs" to the number of single nucleotide mutational events described thus far in *ABCC6* gene and their contribution to the total number of disease-causing mutations in PXE and GACI diseases.

I have contributed to the generation of homology models representing the inward-facing and the outward-facing conformations of the ABCC6 protein. We analyzed the distribution of disease-associated missense mutations on predicted functional and structural surfaces of the protein. We detected the significant clustering of missense mutations on the ABC-ABC and the ABC-ICL contact surfaces. I have also investigated and excluded the potential role of mutational hot spots in the observed clustering.

Arguing the results of Kelly et al., I also discuss the predictivity of homology models, e.g. if homology models are useful in forecasting functional/structural consequences of individual amino acid replacements in the protein structure.

I have also investigated the structure and function consequences of mutations affecting the protein structure by expressing and analyzing four disease-associated missense mutants, S1121W, T1301I, Q1347H and R1459C, of the ABCC6 protein *in vitro*. As part of a large collaborative work I characterized the transport function and the *in vitro* subcellular targeting of these mutants. I have identified them as transport active but mis-localized variants. These mutants served as proper candidates for *in vitr*o and *in viv*o folding correction experiments. Results of these experiments may serve as first steps toward allele specific therapy in PXE and GACI diseases in which no efficient therapy exists.

VIII. ÖSSZEFOGLALÁS

Az ABC (ATP Binding Cassette) transzporterek a nagy transzmembrán fehérjék családjába tartoznak. Minimális funkcionális egységüket két katalitikus, ATP hasító ABC és két a szubsztrát transzlokációs csatornát kialakító transzmembrán domén alkotja. Pontos térszerkezeti adat, kristálykoordináták csak néhányuk esetén állnak rendelkezésre. A transzmembrán és az ABC domének rigid intracelluláris hurkokon keresztül kapcsolódnak, amelyek az ATP hidrolízise során bekövetkező konformációs átrendeződéseket közvetítik a szubsztrát transzlokációs csatorna felé.

Az ABCC6 fehérje génjét érintő mutációkat két kötőszöveti kalcifikációval járó betegség, a pseudoxanthoma elasticum (PXE) és a generalized arterial calcification of infancy (GACI) hátterében is azonosították. Az ABCC6 fehérje fiziológiás szerepéről, illetve a betegségek hátterében meghúzódó patológiás folyamatokról nagyon keveset tudunk. Az ABCC6 fehérje elsősorban a hepatociták bazolaterális membránjában található, míg a betegség tüntei által érintett szövetekből szinte alig kimutatható. Fiziológiás elhelyezkedésének megfelelően, illetve állatmodeleken végzett transzplantációs kísérletek alapján valószínűsíthető, hogy az ABCC6 egy máig ismeretlen metabolit transzportját végzi a májból a vér felé, amely a keringési rendszeren keresztül a perifériás szövetek kalcifikációs folyamatainak megakadályozásában tölt be fontos szerepet. Egy másik egygénes Mendeli betegség, a „PXE-like" szindróma PXE-hez nagyon hasonló kötőszöveti tünetei és a betegség hátterében álló GGCX (gamma-glutamyl carboxylase) enzim-defektus biokémiai hátteréből kifolyólag felmerült hogy az ABCC6-nak szerepe lehet K-vitamin vegyületek, elsősorban anionos K-vitamin konjugátumok májból való transzportjában.

In vitro kísérleteim nem támasztották alá ezt a hipotézist, megállapítottam, hogy az ABCC6 transzprter feltehetőleg nem vesz részt a VK3GS, egy GSH-konjugált K-vitamin származék, májból való transzportjában, azaz feltehetően nem ez a vegyület áll az ABCC6 függő kalcifikációs folyamatok hátterében. Mások *in vitro* és *in vivo* eredményei is témogatták megállapításomat.

ABCC6 gén azonosítása óta, több, mint 400 szekvencia variációt írtak le a génben. A mutációk nagy száma ellenére mindezidáig nem sikerült genotípus-fenotípus összefüggéseket feltárni. Ilyen nagy számú variáns esetén *in vitro* illetve *in vivo*

módszerekkel nem tisztázható valamennyi szekvenciaváltozat fehérje szerkezetre illetve funkcióra gyakorolt hatása. A szekvencia variánsokat és a rendelkezésre álló *in vitro* illetve *in vivo* adatokat, betegség asszociált gének esetén a mutációkat hordozó betegek klinikai adatait rendszerező adatbázisok kiemelkedő fontosságúak.

Kutatócsoportunkban elkészítettünk egy ABCC6 mutációs adatbázis, amely nagymértékben hozzájárult az online elérhető „LOVD ABCC6" adatbázis létrejöttéhez (`http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6`).

Az általunk készített ABCC6 homológiai model, amely az online „LOVD ABCC6" adatbázis felületén is elérhető, lehetőséget nyújt az egyes mutációk térszerkezetben való elhelyezésére, kiindulásul szolgálhat misszensz mutációk a fehérje szerkezet/funkció változásaira kifejtett hatásának tanulmányozásához. Vizsgálataink során megállapítottuk, hogy az ABCC6 fehérje betegség okozó missszensz mutációinak fehérjeszerkezetben való eloszlása nem homogén, a modellünk által jósolt funkcionális és struktúrális felszínek a teljes fehérjeszerkezethez képest érzékenyebbek az aminosavcserét okozó mutációkra. Ezen megfigyelésünk egyfajta genetikai bizonyítéknak is tekinthető ezen felszínek kiemelt fontosságát illetően.

In vitro transzport aktivitási kísérletek, illetve *in vitro* és *in vivo* modelek amelyekben a fehérje lokalizációját/érését/stabilitását vizsgálhatjuk alapvető rendszerek az egyes mutánsok fehérje aktivitásra, illetve szerkezetre gyakorolt hatásának vizsgálatában. Egy nemzetközi együttműködés keretein belül *in vitro* transzport aktivitási kísérletekben és emlős sejtvonalakban *in vitro* lokalizációs vizsgálatokban jellemeztem négy gyakori PXE okozó ABCC6 mutánst. Vizsgálataink célja olyan transzport-aktív, betegség okozó mutációk azonosítása volt, ahol a betegségeket feltehetőleg a fehérje mutáció következtében megváltozott konformációja, és az ennek következtében kialakult lokalizációs defektusok okozzák. Távlati célunk kémia chaperon molekulák alkalmazásával szerkezeti korrekción alapuló terápia kifejlesztése; azaz ezek a mutánsok egy jövőbeli, szerkezeti korrekción alapuló allélspecifikus terápia kiindulópontját képezhetik PXE és GACI betegségekben.

IX. PUBLICATIONS

Research papers

Fülöp K, Barna L, Symmons O, Závodszky P, Váradi A. (2009) Clustering of disease-causing mutations on the domain-domain interfaces of ABCC6. *Biochem Biophys Res Commun* 379, 706-9.

Arányi T, **Fülöp K**, Symmons O, Pomozi V, Váradi A. (2011) Predictable difficulty or difficulty to predict. *Protein Sci*. 20, 1-3.

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Le Saux O, **Fülöp K**, Yamaguchi Y, Iliás A, Szabó Z, Brampton CN, Pomozi V, Huszár K, Arányi T, Váradi A. (2011) Expression and in vivo rescue of human ABCC6 disease-causing mutants in mouse liver. *PLoS One* 6, e24738.

Pomozi V, Brampton C, **Fülöp K**, Chen LH, Apana A, Li Q, Uitto J, Le Saux O, Váradi A. (2014) Analysis of pseudoxanthoma elasticum-causing missense mutants of ABCC6 in vivo; pharmacological correction of the mislocalized proteins. *J Invest Dermato*l 134, 946-53.

Reviews

Váradi A, Szabó Z, Pomozi V, de Boussac H, **Fülöp K**, Arányi T. (2011) ABCC6 as a target in pseudoxanthoma elasticum. *Curr Drug Targets* 12, 671-82

Arányi T, Bacquet C, de Boussac H, Ratajewski M, Pomozi V, **Fülöp K**, Brampton CN, Pulaski L, Le Saux O, Váradi A. (2013) Transcriptional regulation of the ABCC6 gene and the background of impaired function of missense disease-causing mutations. *Front Genet*. 4, 27.

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ABCC6 homepage – LOVD at NCBI – Leiden Open Variation Database

LOVD at NCBI

ABCC6 - ATP-binding cassette, sub-family C (CFTR/MRP), member 6 (ABCC6)

Curators: Sharon Terry and Tim Hefferon

LOVD v.2.0 Build 35 [Current LOVD status]

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ABCC6 homepage Switch gene

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General information	
Gene name	ABCC6 - ATP-binding cassette, sub-family C (CFTR/MRP), member 6
Gene symbol	**ABCC6**
Chromosome Location	16p13.1
Database location	ncbivariation
Curator	Sharon Terry and Tim Hefferon
PubMed references	View all (unique) PubMed references in the ABCC6 database
Date of creation	June 13, 2008
Last update	August 27, 2013
Version	**ABCC6 130827**
Add sequence variant	Submit a sequence variant
First time submitters	Register here
Reference sequence file	Genomic reference sequence for describing sequence variants
Genomic refseq ID	NG_007558.2
Transcript refseq ID	NM_001171.5
Total number of unique DNA variants reported	**288**
Total number of individuals with variant(s)	**496**
Total number of variants reported	**676**
Subscribe to updates of this gene	

Graphical displays and utilities	
Summary tables	Summary of all sequence variants in the ABCC6 database, sorted by type of variant (with graphical displays and statistics)
UCSC Genome Browser	Show variants in the UCSC Genome Browser (compact view)
Ensembl Genome Browser	Show variants in the Ensembl Genome Browser (compact view)
NCBI Sequence Viewer	Show distribution histogram of variants in the NCBI Sequence Viewer

Sequence variant tables	
Unique sequence variants	Listing of all unique sequence variants in the ABCC6 database, without patient data
Complete sequence variant listing	Listing of all sequence variants in the ABCC6 database
Variants with no known pathogenicity	Listing of all ABCC6 variants reported to have no noticeable phenotypic effect (note: excluding variants of unknown effect)
Download table	Download the full sequence variant table of the ABCC6 database in tab-delimited text format.

Search the database	
By type of variant	View all sequence variants of a certain type
Simple search	Query the database by selecting the most important variables (exon number, type of variant, disease phenotype)
Advanced search	Query the database by selecting a combination of variables
Based on patient origin	View all variants based on your patient origin search terms

Links to other resources	
Entrez Gene	368
OMIM - Gene	603234
OMIM - Disease	PXE
HGMD	ABCC6
GeneTests	ABCC6
External link #1	PXE International
External link #2	GeneReviews
External link #3	ABCC6 Homology Model of Human MRP6 Protein (3D)
External link #4	ABCC6 Membrane Topology Model (2D)
External link #5	NCBI MRP6 HomoloGene
External link #6	NG_007558.2 in sequence Viewer
External link #7	ABCC6 cDNA Reference Sequence
External link #8	ABCC6 in NCBI's Map Viewer

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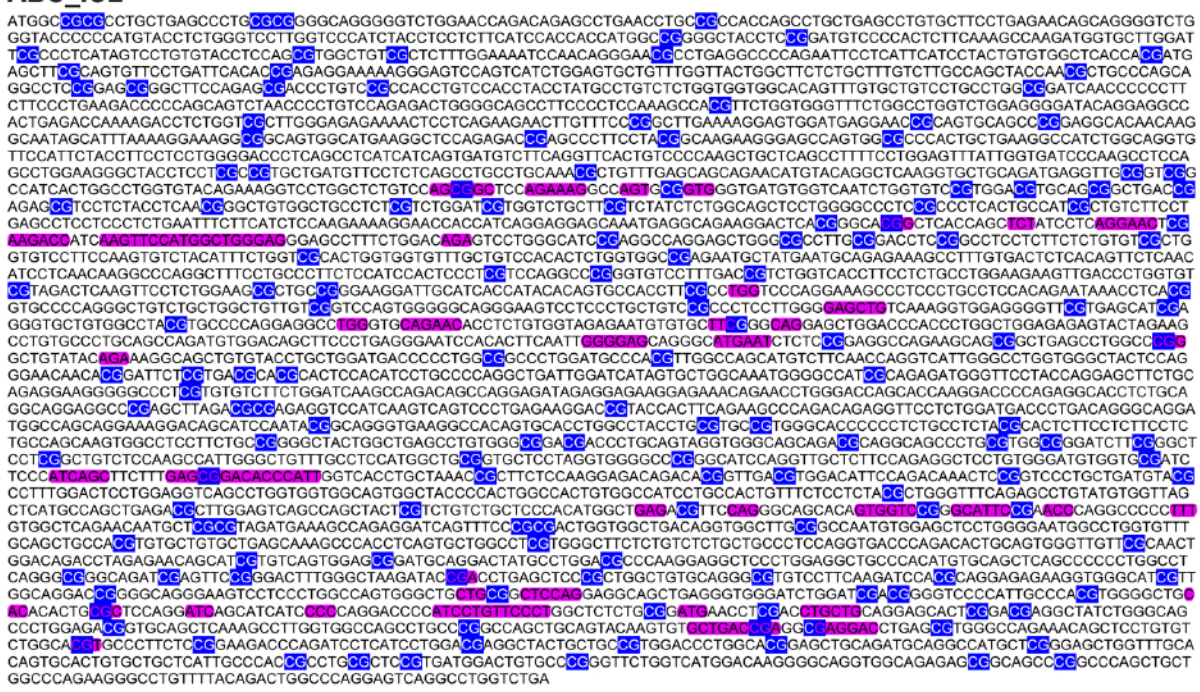
http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6

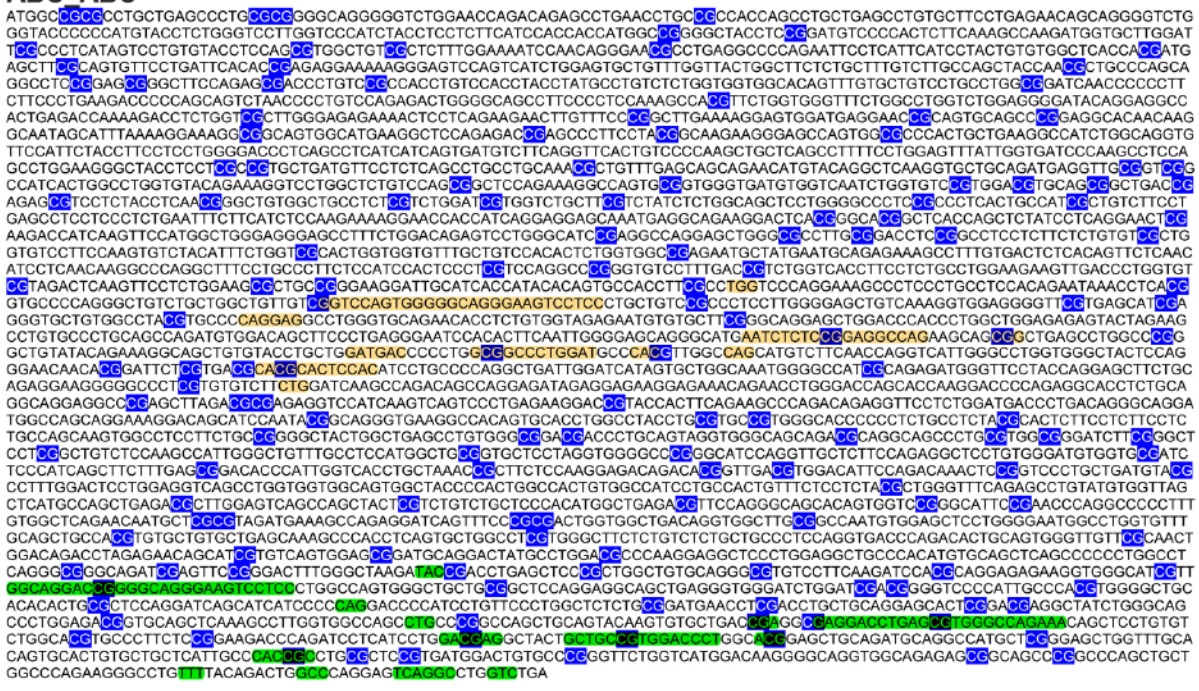
Supp.Fig.1: Screenshot of the ABCC6 LOVD homepage. Tables that include data collected and catalogued with the help of researchers of our group (Orsolya Simmons, Krisztina Fülöp, Tamás Arányi and András Váradi) are highlighted in orange.

Supp.Fig.2

ABC_ICL

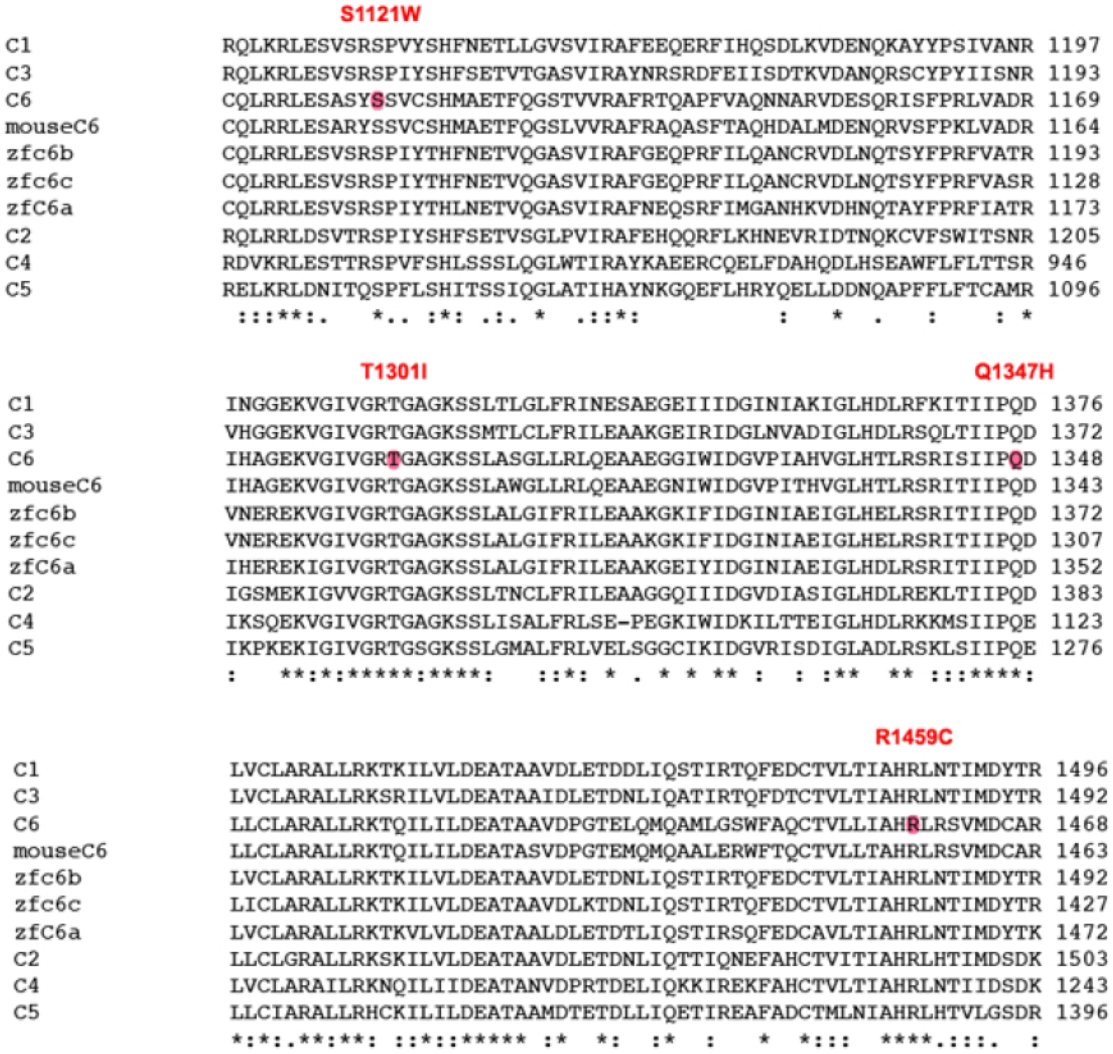


ABC_ABC



Supp.Fig.2: The distribution of CpG dinucleotides within the coding sequence. Nucleotides coding for amino acid residues involved in the ABC-ICL contacts are highlighted in purple; those involved in the ABC-ABC contacts are highlighted in orange and green. CpG dinucleotides are indicated with blue.

Supp.Fig.3



Supp.Fig.3: Protein sequence alignments of human ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, mouse Abcc6, zebrafish Abcc6a, Abcc6b, and Abcc6c proteins were generated by ClustalW2 program using the default settings. The investigated four PXE associated missense mutants of human ABCC6, S1121W, T1301I, Q1347H, and R1459C are indicated with red. Abbreviations are as follows: C1: ABCC1; C2: ABCC2; C3: ABCC3; C4: ABCC4; C5: ABCC5; C6: ABCC6; mouseC6: mouse Abcc6; zfc6a: zebrafishAbcc6a; zfc6b: zebrafishAbcc6b; zfc6c: zebrafishAbcc6c.

Supp.Fig.4

From: **Andras Varadi**
Subject: ABCC6 c.1838T>G p.(V614A)
Date: February 19, 2014 11:35:09 AM GMT+01:00
To: renieri@unisi.it
Cc: Andras Varadi , Krisztina Fulop

Hide
Inbox - Enzim

Dear Dr Reineri,

We are working on extending the ABCC6 variation database (http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6) and are going to incorporate the sequence variation(s) you reported (Meloni et al, 2001).

You report on a new non pathogenic alteration of ABCC6 protein, c.1838T>G p.(V614A) in the paper Meloni et al., 2001. According to the reference cDNA sequence: http://www.ncbi.nlm.nih.gov/nucleotide/NM_001171.5?sendto=t&extrafeatpresent=1

c.1837_1839 is 'GGT' and codes for p.Gly613. Please confirm the cDNA or amino acid alteration.

Thank you for your kind help,

Sincerely,

Krisztina Fülöp and András Váradi

Laboratory of Active Transport Proteins
Institute of Enzymology, RCNS
Hungarian Academy of Sciences
Phone: 361-2793128, Fax: 361-4665465
varadi@enzim.hu

Supp.Fig.4: A representative formal letter that was sent to the corresponding outhors.

Supp.Table1

A	B	C	D	E	F	G	H	I	
sequence variation at DNA level	sequence variation at protein level	Mutation/ Polym.	type of mut. (exonic intronic)	genomic pos. (according to ENSG00000091262.10; 2013. July)	genomic region affected	protein region affected	C>T at 5'CpG	references (allele frequency data and comments)	
c.1-43A>G		P?	i	16317334	5'flanking			RamsayM 2009 (!? Found in 1/24 PXE patient, do not mention if heteroz. or homoz., in mixed South African pop.)	1
c.1-219A>C		P	i	16317510	5'flanking			Pulkkinen2001 (freq:0.950/0.050), also present in Psi1 - gene conv?, Schulz2006 (1/122 in PXE, 14/236 in controls)	2
c.1-132C>T		P	i	16317423	5'flanking		+	Pulkkinen2001 (freq:0.953/0.047), Schulz2006 (freq:2/122 in PXE, 13/236 in controls)	3
c.1-127C>T		P	i	16317418	5'flanking		-	Pulkkinen2001 (freq:0.857/0.143), Schulz2006 (freq: 12/122 in PXE, 34/236 in controls)	4
c.37-55G>A		P?	i	16315743	I1		-	RamsayM 2009 (!? Found in 1/24 PXE patient, do not mention if heteroz. or homoz., in mixed South African pop.)	5
c.37-1G>A		M	i, pot. splice	16315689	I1		-	Schulz2006	6
c.105delA	p.S37fsX80	M	del, fs	16315620	E2	TMH1-TMH2		Miksch 2005 (1/170)	7
c.113G>C	p.W38S	M	m	16315612	E2	TMH1		Schulz2006	8
c.117A>G	p.V39V	P	s	16315608	E2	TMH1		LeSaux2001, also present in Psi2	9
c.175_179del	p.G58fs	M	del, fs	16315550_16315546	E2	ICL1		Vanakker (3/76)	10
c.179_187del	p.R60_Y62del	M	del	16315546_16315538	E2	ICL1		Pulkkinen 2001;Miksch 2005 (1/170) in frame del; Chassaing2005, Bergen2007, Hu2003c	11
c.179_195del		M	del, fs	16315546_16315530	E2	ICL1		LeSaux2001 (0.9% in EU, 0 in US), Chassaing2005, Bergen2007, Hu2003c	12
c.182G>A	p.G61D	P	m	16315543	E2	ICL1	-	LeSaux2001 (although called a "neutral variant" this nucleotide change is only present in 1/244 alleles and in none of the control chromosomes), LeSaux2002 (1/48 in PXE, no control patients, found in a patient homozygous for R1339C, but why does this mean this is a polymorphism?!)	13
c.191G>A	p.R64Q	?	m	16315534	E2	ICL1	+	LeSaux2001, also present in Psi2	14
c.220-1G>C		M?	i, pot. splice	16313805	I2			Pfendner 2007	15
c.220_222del	p.V74del	M	del	16313804_16313802	E3	TMH2		Chassaing 2004 also a splice mutation since this is the first codon of exon 3, Chassaing2005, Bergen2007	16
c.345+12T>C		P	i	16313667	I3			Miksch2005 (intronic duplication)	17
c.345+26C>T		P	i	16313653	I3		+	Miksch2005, RamsayM 2009 (!? Found in 1/10 white and 3/10 black healthy south african person, do not mention if heteroz. or homoz., in mixed South African pop.)	18
c.346-6G>A		P/M	i, pot. splice	16313545	I3		+	Miksch2005 (P), Pulkkinen2001 (P), Schulz2006 (M - affects splicing)	19
c.373G>T	p.E125X	M	n	16313512	E4	ICL2_		Miksch 2005 (1\170)	20
c.373G>A	p.E125K	M	m	16313512	E4	ICL2	+	Vanakker2008 (1/76)	21
c.386G>A	p.G129E	M	m	16313499	E4	ICL2	-	Miksch 2005 (1\170)	22
c.450_451insC	p.A151Rfs	M	ins, fs	16313435_16313434	E4	ECL3_		Nitschke 2012	23
c.473C>T	p.A158V	P?	m, pot.splice	16313412	E4	ECL3	+	Schulz2005b, could also be a frameshift, although they do not mention it	24
c.474+13G>A		P	i	16313398	I4		+	Miksch2005(intronic duplication)	25
c.474+43C>T		P	i	16313368	I4		-	Miksch2005(intronic duplication)	26
c.475-76A>C		P	i	16308382	I4			Miksch2005(intronic duplication)	27
c.475-45C>T		P	i	16308351	I4		-	Miksch2005	28
c.475-22T>C		P	i	16308328	I4			Miksch2005(intronic duplication)	29
c.496C>T	p.R166C	M	m	16308285	E5	ECL3	+	MartinL 2008 (found in a carrier, who has symptome, that closely mimic PXE, also reports on a PXE patient having c.496C>T, in compound heteroz. state with c.ABCC6del, but do not describes the patient in this paper, no controlls)	30
c.549G>A	p.L183L	P	s	16308232	E5	TMH5	-	LeSaux2000, Miksch2005	31
c.595C>T	p.Q199X	M	n	16308186	E5	ICL3	-	Yoshida2005, Chassaing2005	32
c.600+23C>T		P	i	16308158	I5		-	Miksch2005	33
c.619G>A	p.G207R	P ?	m	16306085	E6	ICL3	-	LeSaux2001 (although called a "neutral variant" this nucleotide change is only present in 1/244 alleles and in none of the control chromosomes) Miksch2005, Pulkkinen2001, LeSaux2002 (2/48), Miksch2005, RamsayM 2009 (!? Found in 6/24 south african PXE patient, do not mention if heteroz. or homoz.)	34
c.645G>A	p.T215T	P	s	16306059	E6	ICL3	+		35
c.662+12C>T		P	i	16306030	I6		+	Miksch2005, Pulkkinen2001	36
c.662+114T>C		P	i	16305928	I6			Pulkkinen2001 (also a mutation in Psi1 - gene conversion?)	37
c.662+298C>G		P	i	16305744	I6			Pulkkinen2001	38
c.662+305T>G		P	i	16305737	I6			Pulkkinen2001	39
c.662+403T>A		P	i	16305639	I6			Pulkkinen2001	40
c.676G>A	p.G226R	M	m	16302703	E7	ICL3	-	Chassaing2005, Bergen2007	41
c.681C>G	p.Y227X	M	n	16302698	E7	ICL3		Chassaing 2005, Meloni2001, Bergen2007	42
c.708_709dupCT	p.W237Sfs*21	M	dup	16302671_16302670	ICL3	ICL3		Tan2012 (case report, homozygous mutation in this patient, born from consanguineous parents, no controls)	43
c.724G>T	p.E242X	M	n	16302655	E7	ICL3		Pfendner 2007 (1/542)	44
c.754C>T	p.L252F	M	m	16302625	E7	ICL3	-	Schulz2006 (not found in 200 control alleles, relatively conserved position, but present together with 2 further mutations in the PXE patient!)	45
c.793A>G	p.R265G	P ?	m	16302586	E7	ICL3		LeSaux2001 (not present in 200 normal chr), potentially also splice change, Pulkkinen2001 (freq:0.914/0.086), also present in Psi1 - gene conv?, Schulz2005b, Miksch2005, RamsayM 2009 (!? Found in 1/10 white healthy south african person, do not mention if heteroz. or homoz.)	46
c.794+36A>C		P	i	16302549	I7			Miksch2005(intronic duplication)	47
c.841A>G	p.K281E	P ?	m	16297424	E8	ICL3		LeSaux2001 (reported as silent nucleotide, but found by chance, not tested in control chr - could be pseudogene sequence based on consensus seq.), LeSaux2002 (2/48, no segregation with PXE), Miksch2005(reports this mutation as found in one of the pseudogenes)	48
c.855C>T	p.T285T	P	s	16297410	E8	ICL3	+	LeSaux2002 (found by chance, 2/48 in PXE, no controls!), Miksch2005(reports this mutation as found in pseudogene1)	49
c.865_889del		M	del, fs	16297400_16297376	E8	ICL3		LeSaux2002 (found in a heterozygous state in an apparently healty (control) person)	50
c.938_939insT		M	ins, fs	16297327_16297326	E8	TMH6		LeSaux2001 (1/202), Chassaing2005, Bergen2007, LeSaux2002 (1/34 in Afrikaner, 1/14 in other SA), Hu2003c	51
c.951C>A	p.S317R	M	m	16387314	E8	TMH6		Miksch 2005 (1/170)	52
c.951C>G	p.S317R	M	m	16297314	E8	TMH6		Pfendner 2007 (2/542);	53
c.955A>G	p.I319V	P ?	m	16297310	E8	TMH6		LeSaux2001 (reported as silent nucleotide, but found by chance, not tested in control chr - could be pseudogene sequence based on consensus seq.), LeSaux2002 (2/48 - no segregation with PXE), Miksch2005 (reports this mutation as found in one of the pseudogenes)	54
c.960delC	p.I320fs	M	del, fs	16297305	E8	TMH6		Chassaing 2005; Meloni 2001, Bergen2007, Hu2003c	55
c.993G>T	p.L331L	P	s	16297272	E8	ECL4		Miksch2005, RamsayM 2009 (!? Found in 1/10 black healthy south african person, do not mention if heteroz. or homoz.)	56
c.998+2_998+3delTG		M	i, pot. splice	16297264_16297265	I8			Chassaing2005, Bergen2007	57
c.998+13C>T		P	i	16297254	I8		-	RamsayM 2009 (!? Found in 1/10 black healthy south african person, do not mention if heteroz. or homoz.)	58
c.1064T>G	p.L355R	M	m	16295970	E9	TMH7		Miksch 2005 (1/170), Nitschke2012	59
c.1077A>G	p.S359S	P	s	16295957	E9	TMH7		Miksch2005 (also found in pseodog.1), Cai2001 (in LD with 1132C>T, sometimes also 1141T>C)	60
c.1087C>T	p.Q363X	M	n	16295947	E9	TMH7	-	Pfendner 2007 (1/541)	61
c.1088_1120del	p.363_374QTLF EQQNMYRdel	M	del	16295946_16295914	E9	TMH7-ICL4		Chassaing 2004 in frame deletion, Chassaing2005, Bergen2007, MartinL 2008	62
c.1091C>G	p.T364R	M	m	16295943	E9	TMH7		Pulkkinen 2001; Gheduzzi 2004 (1/98); Hu 2003; Miksch 2005 (1/170), Chassaing2005, Bergen2007, Hu2003c	63
c.1091C>T	p.T364M	M	m	16295943	E9	TMH7	+	Vanakker2008	64
c.1108A>G	p.N370D	M	m	16295926	E9	TMH7		Miksch 2005 (2/170)	65
c.1132C>T	p.Q378X	M	n	16295902	E9	ICL4	-	Pfendner 2007 (4/542); Cai 2001 (possibly in LD with 1077A>G; 1141T>C); Gheduzzi 2004 (5/98); Miksch 2005 (6/170); Chassaing2005, Bergen2007, Schulz2006, Hu2003c, Hu2003b, Schulz2005b, LaRusso2010 (3.8% of mediterraneans, 9.1% of scandinavianmutant alleles)	66
c.1141T>C	p.L381L	P	s	16295893	E9	ICL4		Cai2001 (in LD with 1077A>G; 1132C>T, possible gene conversion), Schulz2005b, Miksch2005(reports this mutation as found in ABCC6 and in pseudogene1)	67
c.1144C>T	p.R382W	M	m	16295890	E9	ICL4	+	Pfendner 2007 (2/542)	68
c.1171A>G	p.R391G	M	m	16295863	E9	ICL4		Pfendner 2007 (3/542); Pulkkinen 2001; Chassaing 2004; Miksch 2005 (2/170); Gotting 2004, Chassaing2005, Bergen2007, Ringpfeil2006, Schulz2006, Nitschke2012, Costrop2010	69

c.1176G>C	p.K392N	M	m	16295858	E9	ICL4		Pfendner 2007 (1/542), also a splice mutation since this position is within the last codon of exon 9	70
c.1176+6C>T		P	i, pot. splice	16295852	I9		-	Miksch2005	71
c.1192A>G	p.S398G	M	m	16292024	E10	ICL4		Miksch 2005 (1/170)	72
c.1194C>G	p.S398R	M	m	16292022	E10	ICL4		Vanakker2008 (1/76)	73
c.1233T>G	p.N411K	M	m	16291983	E10	ICL4		LeSaux 2001 (0 in EU, 1.4% in US), Chassaing2005, Bergen2007, Gheduzzi2004 (1/98), Hu2003c	74
c.1233T>C	p.N411N	P	s	16291983	E10	ICL4		Miksch2005, HapMap, LeSaux2002 (20/48 in PXE, no controls), Morcher2003, Wang2001 (0.42 in Caucasians), Gheduzzi2004, Meloni2001, Schulz2005b, Miksch2005, RamsayM 2009 (!? Found in 2/10 black 1/10 white healthy south african person, and in 1/24 PXE patient do not mention if heteroz. or homoz.)	75
c.1244T>C	p.V415A	M	m	16291972	E10	ICL4		Schulz2006 (not found in 200 control alleles, relatively conserved position, but present together with 2 further mutations in the PXE patient!)	76
c.1245G>A	p.V415V	P	s	16291971	E10	ICL4	-	Hu2003b (est. freq. 33%), Miksch2005, LeSaux2002 (20/45 in PXE, no controls), Morcher2003, Wang2001 (0.42 in Caucasians), Gheduzzi2004, Schulz2005b, Miksch2005, RamsayM 2009 (!? Found in 2/10 black healthy south african person, do not mention if heteroz. or homoz.)	77
c.1249G>A	p.V417M	P/M	m	16291967	E10	ICL4	+	RamsayM 2009 (!? Found in 5/24 PXE patient, do not mention if heteroz. or homoz., in mixed South African pop.)	78
c.1256G>A	p.R419Q	M?	m	16291960	E10	ICL4	+	Sato et al 2009 (associates with angioid streaks, in a study collecting patients with AS but not with the full diagnosys of PXE, 18/108 AS allele and 0/300 control allele)	79
c.1264G>A	p.E422K	M?	m	16291952	E10	ICL4	+	Sato et al 2009 (associates with angioid streaks ,AS, in a study collecting patients with AS but not with the full diagnosys of PXE, 1/108 AS allele and 0/300 control allele)	80
c.1283A>G	p.N428S	P	m	16291933	E10	TMH8		Sato et al 2009 (found in healthy individuals (2/300 control allele, 2 heterozygotes) in a study collecting patients with AS but not with the full diagnosys of PXE)	81
c.1312G>A	p.V438M	P	m	16291904	E10	TMH8	+	Sato et al 2009 (found in healthy individuals (1/300 control allele, 1 heterozygote) in a study collecting patients with AS but not with the full diagnosys of PXE)	82
c.1318T>G	p.C440G	M	m	16291898	E10	TMH8		Gheduzzi 2004 (1/98); Chassaing 2005, Bergen2007	83
c.1338+7C>G		P	i	16291871	I10			Miksch2005, RamsayM 2009 (!? Found in 5/10 black healthy south african person, do not mention if heteroz. or homoz.)	84
c.1338+20C>G		P	i	16291858	I10			Miksch2005	85
c.1338+62G>C		P	i	16291816	I10			Miksch2005	86
c.1338+1383T>C		P	i	16290495	I10			HapMap	87
c.1338+1501G>A		P	i	16290377	I10		-	HapMap	88
c.1338+1548C>T		P	i	16290330	I10		+	HapMap	89
c.1338+2152G>T		P	i	16289726	I10			HapMap	90
c.1338+2281A>C		P	i	16289597	I10			HapMap	91
c.1338+2346A>G		P	i	16289532	I10			HapMap	92
c.1339-2347C>G		P	i	16289126	I10			HapMap	93
c.1339-1977G>A		P	i	16288756	I10		-	HapMap	94
c.1344G>A	p.L448L	P	s	16286774	E11	ECL5	-	RamsayM 2009 (!? Found in 2/10 black healthy south african person, do not mention if heteroz. or homoz.)	95
c.1363G>C	p.A455P	M	m	16286755	E11	TMH9		Uitto 2001, LeSaux2001, Chassaing2005, Hu2003c, Ringpfeil2001	96
c.1388T>A	p.L463H	M	m	16286730	E11	TMH9		Pfendner 2007 (1/542)	97
c.1396A>T	p.N466Y	M	m	16286722	E11	TMH9		Vanakker2008 (1/76)	98
c.1424A>T	p.H475L	M?	m	16286694	E11	ICL5		MartinL 2008 (found in a carrier, who has symptome, that closely mimic PXE, no controlls, does not describe the proband in the paper)	99
c.1431+73G>C		P	i	16286614	I11			LeSaux2002 (2/48)	100
c.1432-215A>G		P	i	16284439	I11			HapMap	101
c.1432-48G>A		P	i	16284272	I11		-	HapMap, RamsayM 2009 (!? Found in 8/10 white healthy south african person, and in 7/24 PXE patient, do not mention if heteroz. or homoz.)	102
c.1432-45C>A		P	i	16284269	I11			LeSaux2002 (1/48)	103
c.1432-45C>T		P	i	16284269	I11		-	RamsayM 2009 (!? Found in 2/10 white healthy south african person, do not mention if heteroz. or homoz.)	104
c.1432-41A>G		P	i	16284265	I11			LeSaux2000, HapMap, Miksch2005, LeSaux2002 (5/48)	105
c.1432-41A>C		P	i	16284265	I11			RamsayM 2009 (!? Found in1/10 black 2/10 white healthy south african person, a	106
c.1432-22C>A		P	i	16284246	I11			LeSaux2002 (1/48)	107
c.1460G>A	p.R487Q	M	m	16284196	E12	ICL5	+	Schulz2006	108
c.1484T>A	p.L495H	M	m	16284172	E12	ICL5		Pfendner 2007 (1/542); Miksch 2005 (1/170)	109
c.1491C>A	p.N497K	M	m	16284165	E12	ICL5		Schulz2006	110
c.1498A>C	p.T500P	M	m	16284158	E12	ICL5		Vanakker2008 (1/76)	111
c.1505A>T	p.K502M	M	m	16284151	E12	ICL5		Chassaing 2005, Bergen2007	112
c.1540G>A	p.V514I	P ?	m	16284116	E12	ICL5	-	Miksch2005 (cosegregates with other PXE mutations in linkage disequilibrium)	113
c.1552C>T	p.R518X	M	n	16284104	E12	ICL5	+	Pfendner 2007 (2/542); Chassaing 2004; Gheduzzi 2004 (11/98); Meloni 2001; Miksch 2005 (1/170); Chassaing2005, Bergen2007, Ringpfeil2006, Schulz2006, Hu2003c, Hu2003b, Nitschke2012, Costrop 2010	114
c.1553G>A	p.R518Q	M	m	16284103	E12	ICL5	+	Pfendner 2007 (4/542); Chassaing 2004; Gheduzzi 2004 (5/98); LeSaux 2000; Miksch 2005 (4/170); Ringpfeil 2001, LeSaux 2001 (0.9% in EU, 1.4% in US); Uitto 2001, Chassaing2005, Bergen2007, LeSaux2002 (1/14), Hu2003c, Faria 2013, RamsayM 2009 (3/95 PXE alleles, 0.032 in mixed South African pop.), Costrop 2010	115
c.1563G>C	p.E521D	M	m	16284093	E12	ICL5		Vanakker2008 (1/76)	116
c.1574_1575insG	p.L525fsX73	M	ins, fs	16284082_16284081	E12	ICL5		Schulz2006	117
c.1603T>C	p.S535P	M	m	16284053	E12	TMH10		Pfendner 2007 (1/542)	118
c.1635+48C>T		P?	i	16283973	I12		+	RamsayM 2009 (!? Found in 7/24 PXE patient, do not mention if heteroz. or homoz.) here they refer as it was descibed in Miksch 2005 but it was not, there was most probably an errata in Miksch 2005	119
c.1636-240G>A		P	i	16283071	I12		+	HapMap	120
c.1636-112C>T		P	i	16282943	I12		+	HapMap	121
c.1652T>C	p.F551S	M	m	16282815	E13	TMH10		Miksch 2005 (1/170)	122
c.1674delC	p.A558fs	M	del, fs	16282793	E13	ECL6_		Vanakker2008 (1/76)	123
c.1685T>C	p.M562T	M	m		E13	ECL6		Plomp 2008 (0/140 control chromosome)	124
c.1703T>C	p.F568S	M	m	16282764	E13	ICL6		LeSaux 2001 (0 in EU, 1.4% in US), Pfendner 2007 (1/542), Chassaing2005, Bergen2007, Ringpfeil2006, Hu2003c	125
c.1760C>G	p.S587C	P?	m	16282707	E13	TMH11		Sato et al 2009 (found in healthy (1/300, 1 heterozygote) and AS (1/108, 1 heterozygote) individuals in a study collecting patients with AS but not with the full diagnosys of PXE)	126
c.1769C>T	p.S590F	M	m	16282698	E13	TMH11	-	Nitschke2012	127
c.1779+381C>T		P	i	16282307	I13		+	HapMap	128
c.1780-86G>T		P	i	16281154	I13			Chassainq2004, HapMap	129
c.1780-29T>A		M	i, pot. splice	16281097	I13			Chassaing 2005, Bergen2007	130
c.1781C>T	p.A594V	M	m	16281067	E14	TMH11	-	Miksch 2005 (2/170) c.1781 is the 2nd nucleotide position of e14 - possible splice mut?	131
c.1798C>T	p.R600C	M	m	16281050	E14	ICL6	+	Pfendner 2007 (1/542), Costrop 2010	132
c.1841T>C	p.V614A	P	m	16281007	E14	ICL6		LeSaux2000, LeSaux2001 (200/244 PXE alleles, 163/200 control chromosomes), HapMap, Chassaing2004, Hu2003b (est.freq.52%), Miksch2005 (no cosegregation with disease), LeSaux2002 (16/48, no segregation with PXE), Wang2001 (0.17 in Chinese; 0.27 in Oji-Cree; 0.32 in South Asians; 0.41 in Africans; 0.45 in Caucasians; 0.56 in Inuit), Gheduzzi2004, Miksch2005,Schulz2006, Schulz2005b, SatoN2009 (studied amongst patients having AS but not full PXE, found in 16/108 AS alleles and 66/150 control alleles), RamsayM 2009 (!? Found in 2/10 black 3/10 white healthy south african person, and in 15/24 PXE patient, do not mention if heteroz. or homoz.)	133
c.1857_1858insC		M	fs	16280991_16280990	E14	ICL6_		Pfendner 2007	134
c.1867+60A>G		P	i	16280921	I14			HapMap	135
c.1867+205C>G		P	i	16280776	I14			HapMap	136
c.1867+576G>A		P	i	16280405	I14		-	HapMap	137
c.1868-823C>T		P	i	16279714	I14		-	HapMap	138
c.1868-392C>T		P	i	16279283	I14		-	HapMap	139
c.1868-157C>G		P	i	16279048	I14			HapMap	140
c.1868_92delG		P	i	16278983	I14			Chassaing2004, Gheduzzi2004	141

c.1868-90G>T		P	i	16278981	I14			Chassaing2004, Gheduzzi2004	142
c.1868-57G>A		P	i	16278948	I14		+	Chassainq2004, Miksch2005, Gheduzzi2004	143
c.1868-5T>G		M	i, pot. splice	16278896	I14			Chassaing 2005, Bergen2007	144
c.1890C>G	p.T630T	P	s	16287869	E15	ICL6		Chassaing2004, Hu2003b (est.freq.22%), Miksch2005, LeSaux2002 (11/48), Morcher2003, Wang2001 (0.6 in Caucasians), Gheduzzi2004, Meloni2001, Schulz2005b, RamsayM 2009 (!? Found in 3/10 black 1/10 white healthy south african person, and in 6/24 PXE patient, do not mention if heteroz. or homoz.)	145
c.1896C>A	p.H632Q	P	m	16287863	E15	ICL6		LeSaux2001 (17/24 PXE alleles, ND in control chromosomes), HapMap, Chassaing2004, Hu2003b (24%), Miksch2005 (in LD with other PXE mutations), LeSaux2002 (11/48, no segregation with PXE), Morcher2003, Wang2001 (0.4 in Caucasians), Gheduzzi2004, Struk2000, Hu2003a (34/32 in PXE, 80/204 in controls), Schulz2005b, SatoN2009 (studied amongst patients having AS but not full PXE, found in 33/108 AS alleles and 65/150 control alleles), RamsayM 2009 (!? Found in 3/10 black 1/10 white healthy south african person, do not mention if heteroz. or homoz.)	146
c.1943+121T>A		P	i	16278695	I15			HapMap	147
c.1943+766G>A		P	i	16278050	I15		-	HapMap	148
c.1944-898C>T		P	i	16277685	I15		-	HapMap	149
c.1944-177G>A		P	i	16276964	I15		-	HapMap	150
c.1944_1965del	p648fsX688	M	del, fs	16276787_16276766	E16	ICL6 (WalkerA)		LeSaux2001, Hu2003b (2/118), Bergen2000, Hu2004 (2/152), Hu2003a, Hu2003c, Ringpfeil2001	151
c.1964A>G	p.Q655R	?	m	16276767	E16	ICL6		Schulz2005a (1/266 in AAA patient, 0 in PXE and controls)	152
c.1967_1989del		M	fs	16276764_16276742	E16	ICL6_		Bergen 2000	153
c.1987G>T	p.G663C	M	m	16276744	E16	ICL6 (WalkerA)		Pfendner 2007 (1/542)	154
c.1990C>T	p.P664S	?	m	16276741	E16	ICL6 (WalkerA)	-	Schulz2005a (0 in PXE/AAA patients, 1/286 in control)	155
c.1994T>C	p.V665A	P?	m	16276737	E16	ICL6 (WalkerA)		HapMap (only described in 1 individual)	156
c.1995delG	p.A667fsX20	M	del, fs	16276736	E16	ICL6 (WalkerA)		LeSaux2001 (0.9% in EU, 0 in US), Hendig2005 (3/152), Chassaing2005, Bergen2007, Schulz2005a (3/108 in PXE patients), Schulz2006, Hu2003c, Schulz2005b	157
c.2018T>C	p.L673P	M	m	16276713	E16	ICL6		LeSaux 2001, Chassaing2005, Bergen2007, LeSaux2002 (1/34 in Afrikaner, 0 in other SA), Hu2003c	158
c.2030T>C	p.L677P	M	m	16276701	E16	ICL6		Miksch 2005 (1/170)	159
c.2070+5G>A		M ?	i, pot. splice	16276656	I16		-	Pfendner 2007 (no control alleles were analyzed)	160
c.2093A>C	p.Q698P	M	m	16276423	E17	ICL6 (Q-loop)		Pfendner 2007	161
c.2097G>T	p.E699D	M	m	16276419	E17	ICL6		Pfendner 2007 (1/542)	162
c.2162G>A	p.W721X	M	n	16276354	E17	ICL6	-	Miksch 2005 (1\170)	163
c.2171G>A	p.R724K	P	m	16276345	E17	ICL6	-	Hendig 2005 (3/260 PXEp. - 2/200 control), Miksch2005 (no cosegregation with disease), Schulz2005a (7/674), Ramsay 2009 (uncorrect protein alteration reported)	164
c.2175A>T	p.V725V	P	s	16276341	E17	ICL6		Hendig 2005 (3/260 PXEp. - 2/200 control), Miksch2005, Schulz2005a (7/674), RamsayM 2009 (Found in 2/10 blacksouth african pop. , do not mention if heteroz. or homoz.)	165
c.2177T>C	p.L726P	M	m	16276339	E17	ICL6		Pfendner 2007 (1/542), RamsayM 2009 1/47 in mixed South African PXE alleles, 0/200 control alleles)	166
c.2224A>G	p.I742V	P	m	16276292	E17	ICL6		Hendig 2005 (3/260 PXEp. - 2/200 control), Miksch2005 (no cosegregation with disease), Schulz2005a (7/674) RamsayM 2009 (Found in 1/10 black south african pop. , do not mention if heteroz. or homoz.)	167
c.2237_2238ins10		M	fs	16276279_16276278	E17	ICL6_		Pfendner 2007	168
c.2245C>T	p.Q749X	M	n	16276271	E17	ICL6	-	Chassaing 2005, Bergen2007, Hu2003a	169
c.2247+22T>G		?	i	16276247	I17			Schulz2005a (0 in PXE patients and controls, 1/266 in AAA patients)	170
c.2247+302C>T		P	i	16275967	I17		+	HapMap	171
c.2247+857C>T		P	i	16275412	I17		-	HapMap	172
c.2248-1384G>C		P	i	16274206	I17			HapMap	173
c.2248-552G>A		P	i	16273374	I17		+	HapMap	174
c.2248-12_11delTT		M	i, pot. splice	16272833_16272834	I17			Hu2003b (reported as splice site mutation, but no further evidence to support this), Chassaing2005, Bergen2007, Hu2004 (1/152)	175
c.2248-2_2248-1del	p.750_805del	M	i, pot. splice	16272823_16272824	I17			Gheduzzi2004 (1/98) - RT-PCR and sequencing show that this mutation results in skipping of exon18	176
c.2252T>A	p.M751K	M	m	16272818	E18	ICL6		Pfendner 2007 (1/542); Hendig 2005 (5/152); Gotting 2004, Schulz2005a (2/108 in PXE patients), Schulz2006	177
c.2263G>A	p.G755R	M	m	16272807	E18	ICL6 (Signature)	+	Pfendner 2007 (2/542)	178
c.2278C>T	p.R760W	M	m	16272792	E18	ICL6 (Signature)	+	Pfendner 2007 (3/542); Schulz 2005a (1/108 in PXE patients); Gotting 2004; Miksch 2005 (1/170); Hendig 2005 (5/260), Schulz2006	179
c.2279G>A	p.R760Q	M	m	16272791	E18	ICL6 (Signature)	+	Vanakker2008 (2/76)	180
c.2293C>T	p.R765W	M	m	16272777	E18	ICL6 (Signature)	+	Vanakker2008 (1/76)	181
c.2294G>A	p.R765Q	M	m	16272776	E18	ICL6 (Signature)	+	Hendig 2005 (4/260); Hu 2003b (1/118); Hu 2004 (1/152); LeSaux 2002; Miksch 2005 (1/170); Schulz 2005a (3/108 in PXE patients); Gotting 2004, Pfendner 2007 (2/542); LeSaux 2001 (0.9% in EU, 0 in US), Chassaing2005, Bergen2007, Schulz2006, Hu2003c, LeBoulanger 2009 (most probably associated with GACI, uncorrectly reported mutation, most probably R765Q)	182
c.2297C>A	p.A766D	M	m	16272773	E18	ICL6 (Signature)		Chassaing 2004, Chassaing2005, Bergen2007	183
c.2304C>A	p.Y768X	M	n	16272766	E18	ICL6		LeSaux 2001;Miksch 2005 (1/170), Chassaing2005, Bergen2007, LeSaux2002 (4/34 in Afrikaner, 0 in other SA), Hu2003c, RamsayM 2009 (8/95 PXE alleles, 0.084 in mixed South African pop.)	184
c.2322delC	p.774Yfs	M	del, fs	16272748	E18	ICL6_		LeSaux2001 (1/74 in US), Chassaing2005, Bergen2007, Hu2003c	185
c.2329G>A	p.D777N	M	m	16272741	E18	ICL6 (WalkerB)	-	Pfendner 2007 (1/542)	186
c.2342C>T	p.A781V	M	m	16272728	E18	ICL6 (WalkerB)	+	Chassaing 2005, Bergen2007	187
c.2379C>G	p.N793L	M	m	16272691	E18	ICL6		Vanakker2008	188
c.2400A>G	p.G800G	P	s	16272670	E18	ICL6		HapMap, RamsayM 2009 (Found in 2/10 black , 1/24 PXE patients in south african pop.do not mention if heteroz. or homoz.)	189
c.2415+147C>T		P	i	16272508	I18		+	HapMap	190
c.2419C>T	p.R807W	M	m	16271480	E19	ICL6	+	Miksch 2005 (1/170) c.2419 is the 4th nucleotide posotion of e19 - possible splice mut?	191
c.2420G>A	p.R807Q	M	m	16271479	E19	ICL6	+	Miksch 2005 (1/170) c.2420 is the 5th nucleotide posotion of e19 - possible splice mut?, Chassaing2005, Bergen2007	192
c.2428G>A	p.V810M	M	m	16271471	E19	ICL6	+	Chassaing 2005; Gheduzzi 2004 (1/98), Bergen2007	193
c.2432C>T	p.T811M	M	m	16271467	E19	ICL6	+	Pfendner 2007 (1/542); Ringpfeil 2006	194
c.2458G>C	p.A820P	M	m	16271441	E19	ICL6		Chassaing 2005; Gheduzzi 2004 (2/98), Bergen2007	195
c.2477T>C	p.L826P	M	m	16271422	E19	ICL6		Plomp 2008 (0/140 control chromosome)	196
c.2490C>T	p.A830A	P	s	16271409	E19	ICL6	-	Hendig 2005 (allele frequency data was not clear); LeSaux 2000; Hu 2003, HapMap, Miksch2005, Ringpfeil2001b, LeSaux2002 (6/48), Morcher2003, Wang2001 (0.08 in Caucasians), Gheduzzi2004, Schulz2006, Schulz2005b, RamsayM 2009 (Found in 3/24 PXE patient in south african pop., do not mention if heteroz. or homoz.)	197
c.2511C>A	p.Y837X	M	n	16271388	E19	ICL6		Hefferon2007	198
c.2524C>T	p.Q842X	M	n	16271375	E19	ICL6	-	Miksch 2005 (1\170)	199
c.2542delG	p.2848fs	M	del, fs	16271357	E19	ICL6		Uitto2001, LeSaux2001, Chassaing2005, Bergen2007, Noji2004, Hu2003c, Ringpfeil2001, according to reference genome there is an A at this position, but this is also a HapMap polymorphism, with A being the minor allele only present in yoruba and japanese, SatoN2009 (associates with angioid streaks, in a study collecting patients with AS but not with the full diagnosys of PXE, 27/108 AS allele and 1/300 control allele), La Russo2010(onlz present in asians, japanese)	200
c.2542A>G	p.M848V	P	m	16271357	E19	ICL6		HapMap, RamsayM 2009 (Found in 3/10 black healthy . 1/24 PXE patient in south african pop, do not mention if heteroz. or homoz.)	201
c2552T>C	p.L851P	M	m	16271347	E19	ICL6		Hendig 2005 (2/260); Schulz2006 (has been described as mutation, but occurs together with 2 definate mutations!)	202
c.2590+405G>A		P	i	16270904	I19		-	HapMap	203
c.2590+459G>A		P	i	16270850	I19		-	HapMap	204
c.2591-331A>G		P	i	16270174	I19			HapMap	205
c.2591-203T>C		P	i	16270046	I19			HapMap	206
c.2591-119C>T		P	i	16269962	I19		-	HapMap	207
c.2631C>A	p.T877T	P	s	16269803	E20	ICL6		Morcher2003(present in several non-PXE people)	208
c.2643G>T	p.R881S	M	m	16269791	E20	ICL6		Pfendner 2007 (1/542)	209

c.2666+133_134insC		P	i	16269634_16269635	I20			Hendig2005 (4/260)	210
c.2666+198T>C		P	i	16269570	I20			HapMap	211
c.2666+499C>T		P	i	16269269	I20		+	HapMap	212
c.266+900C>T		P	i	16268868	I20		+	HapMap	213
c.2695C>G	p.R899G	P ?	m	16267233	E21	ICL6		HapMap	214
				16267140				LeSaux2000 (mutates donor site, lowers splice potential score from 72.1 to 53.8), Uitto2001, Miksch 2005 (5/170), LeSaux2001 (3.4% in EU, 4.1% in US), Hendig2005 (11/260), Chassaing2005, Bergen2007, Schulz2006, Hu2003c, Ringpfeil2001, Schulz2005b, Nitschke2012,	
c.2787+1G>T		M	i, pot. splice		I21			Costrop 2010	215
c.2787+30G>A		P	i	16267111	I21		+	LeSaux2002 (2/48)	216
c.2787+62T>C		P	i	16267079	I21			HapMap	217
c.2787+1427C>G		P	i	16265714	I21			HapMap	218
c.2787+1580G>T		P	i	16265561	I21			HapMap	219
c.2788-706T>C		P	i	16264416	I21			HapMap	220
c.2788-388A>C		P	i	16264098	I21			HapMap	221
c.2788-127A>G		P	i	16263837	I21			HapMap	222
c.2814C>G	p.Y938X	M	n	16263684	E22	ICL6		Pfendner 2007 (1/542)	223
c.2820T>G	p.R940R	P	s	16263678	E22	TMH12		Miksch2005	224
c.2820insC		M	fs	14263678	E22	TMH12_		Pfendner 2007	225
c.2831C>T	p.T944I	M	m	16263667	E22	TMH12	-	Pfendner 2007 (1/542)	226
				16263663				Hendig 2005 (7/240); LeSaux 2002, HapMap, Morcher2003, RamsayM 2009 (Found in 2/10 black healthy, 1/10 white healthy, 1/24 PXE patient in south african pop, do not mention if heteroz. or	
c.2835C>T	p.P945P	P	s		E22	TMH12	-	homoz.)Miksch2005	227
				16263663_16263648				Hendig 2005 (1/108) out of frame deletion that results in 17 new	
c.2835 _2850del16	p.P946fsX17	M	del, fs		E22	TMH12		codons and premature STOP, Schulz2006	228
				16263662				Hendig 2005(3/260 PXEp. - 7/200 control); Morcher 2003 (M), Miksch2005 (P, no cosegregation with disease), Shi2007, Hendig (P, because present in normal pop) vs Morcher (M), RamsayM 2009 (Found in 1/24 PXE patient in south african pop, do not mention if	
c.2836C>A	p.L946I	P	m		E22	TMH12		heteroz. or homoz.)	229
c.2848G>A	p.A950T	M	m	16263650	E22	TMH12	+	Pfendner 2007 (1/542)	230
c.2849C>T	p.A950V	P?	m	16263649	E22	TMH12	-	Sato et al 2009 (found in healthy (1/300, 1 heterozygote) and AS (1/108 1 heterozygote) individuals in a study collecting patients with AS but not with the full diagnosys of PXE)	231
c.2855T>G	p.F952C	M ?	m	16263643	E22	TMH12		Hendig 2005 (1/260) located in cis-position with c.2835-2850del; Schulz2006	232
				16263640				LeSaux2001 (although called a "neutral variant" this nucleotide change is only	
c.2858T>A	p.L953H	P ?	m		E22	TMH12		present in 1/244 alleles and in none of the control chromosomes)	233
c.2891G>C	p.R964P	M	m	16263607	E22	ECL7		Plomp 2008 (0/140 control chromosome)	234
c.2904G>A	p.L968L	P	s	16263594	E22	ECL7	-	Hu2003b (est.freq.20%), Miksch2005	235
c.2943G>T	p.Q981H	M	m	16263555	E22	ECL7		Vanakker2008 (1/76)	236
c.2974G>C	p.G992R	M	m	16263642	E22	ECL7		Chassaing 2005; Schulz 2006; Pfendner 2007, Bergen2007	237
c.2995+142C>T		P	i	16263361	I22		+	HapMap	238
c.2995+931C>T		P	i	16262572	I22		-	HapMap	239
c.2995+1329G>A		P	i	16262174	I22		-	HapMap	240
c.2996-1461G>A		P	i	16261251	I22		-	HapMap	241
c.2996-1380C>G		P	i	16261170	I22			HapMap	242
c.2996-1353G>A		P	i	16261143	I22		-	HapMap	243
c.2996-1213G>A		P	i	16261003	I22		-	HapMap	244
c.2996-1028G>T		P	i	16260818	I22			HapMap	245
c.2996-653T>C		P	i	16260443	I22			HapMap	246
c.2996-637G>A		P	i	16260427	I22		+	HapMap	247
c.2996-552G>A		P	i	16260342	I22		+	HapMap	248
c.3074T>C	p.L1025P	M	m	16259712	E23	ICL7		Vanakker2008 (1/76)	249
				16259698				LeSaux 2001 (1/202), Gheduzzi 2004 (5/98); Chassaing 2005,	
c.3088C>T	p.R1030X	M	n		E23	ICL7	+	Bergen2007, LeSaux2002, Hu2003c	250
c.3105_3107delCTT	p.F1036del	M	del	16259681_16259679	E23	ICL7		Nitschke 2012	251
c.3106_3108delTTT	p.F1036del	M	del	16259680_16259678	E23	ICL7		Miksch 2005 (1/170)in frame del	252
c.3141_3143delTCT	p.F1048del	M	del	16259645_16259643	E23	ICL7		Miksch 2005 (1/170)	253
				16259641				Schulz2006 (not present in 200 control alleles, relatively conserved position, but	
c.3145T>G	p.S1049A	M	m		E23	ICL7		occurs together with 2 further mutations in PXE patient!)	254
c.3168C>A	p.D1056E	M	m	16259618	E23	ICL7		Miksch 2005 (1/170)	255
c.3188T>G	p.L1063R	M	m	16259598	E23	TMH14		Schulz2006	256
c.3190C>T	p.R1064W	P	m	16259596	E23	TMH14	+	Miksch2005 (does not cosegregate with disease), Morcher2003	257
c.3207C>A	p.Y1069X	M	n	16259579	E23	TMH14		Plomp 2008	258
c.3306+531C>T		P	i	16258949	I23		+	HapMap	259
c.3306+650A>G		P	i	16258830	I23			HapMap	260
c.3306+855G>T		P	i	16258625	I23			HapMap	261
c.3307-1124C>T		P	i	16258173	I23		+	HapMap	262
c.3307-897A/C/G/T		P	i	16257946	I23			HapMap	263
c.3307-361G>T		P	i	16257410	I23			HapMap	264
c.3307-3_-38del and insAGA		M	i, pot. splice	16257052_16257087	I23			Miksch2005(1/170)	265
				16257016				Gheduzzi 2004 (1/98); Miksch 2005 (1/170); Schulz 2005a (1/108 in PXE patients); Gotting 2004; Klein 2001, Chassaing2005, Bergen2007, Schulz2006,	
c.3340C>T	p.R1114C	M	m		E24	ICL8	+	Schulz2005b (1/108), Nitschke 2012	266
				16257015				LeSaux 2001 (1/116 in EU), LeSaux2000 (not present in a control panel of 200 chromosomes), Uitto2001, Chassaing2005, Bergen2007,	
c.3341G>C	p.R1114P	M	m		E24	ICL8		Audo2007, Hu2003c, Ringpfeil2001	267
				16257015				Hu2003b (1/118), Götting2004(1/128), Chassaing2005, Bergen2007, Hu2004 (1/152), Schulz2005a (1/108 in PXE patients), Schulz2006,	
c.3341G>A	p.R1114H	M	m		E24	ICL8	+	Schulz2005b (1/108)	268
c.3343_3345del	p.L1115del	?	del	16257013_16257011	E24	ICL8		Bergen2007	269
c.3362C>T	p.S1121L	M	m	16256994	E24	ICL8	+	Miksch 2005 (3/170)	270
c.3362C>G	p.S1121W	M	m	16256994	E24	ICL8		LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007, Hu2003c	271
c.3364delT	p.S1122Lfs	M	fs	16256992	E24	ICL8		Vanakker2008 (1/76)	272
c.3381G>A	p.M1127I	?	m	16256975	E24	ICL8	-	Hefferon2007	273
				16256967				Chassaing 2004; Gheduzzi 2004 (2/98); Hu 2003; Hu 2004;Miksch 2005; Ringpfeil 2000; Schulz 2005a (2/108 in PXE patients); Gotting 2004 (2/128); Klein 2001, Pfendner 2007, Chassaing2005, Bergen2007, Morcher2003,	
c.3389C>T	p.T1130M	M	m		E24	ICL8	+	Schulz2006, Shi2007 (1/32), Schulz2005b (2/108), Martin L2008	274
c.3397G>T	p.G1133C	M	m	16256959	E24	ICL8		Drera2007 (experimentally validated: in trans with R1141X)	275
c.3398G>C	p.G1133A	M	m	16256958	E24	ICL8		Pfendner 2007	276
				16256944				Ringpfeil 2000 (1/16); Miksch 2005 (1/170), Uitto2001, LeSaux2001, Chassaing2005, Bergen2007, Ringpfeil2006, Schulz2006, Hu2003c,	
c.3412C>T	p.R1138W	M	m		E24	ICL8	+	Ringpfeil2001, Shi2007(2/32), HesseRJ 2010	277
				16256943				LeSaux 2001 (0.9% in EU, 1.4% in US); Miksch 2005 (3/170); Ringpfeil 2000 (1/16), LeSaux2000, Uitto2001, Chassaing 2005; Gheduzzi 2004 (1/98); Chassaing 2004, Bergen2007, LeSaux2002 (3/34 in Afrikaner, 0 in other SA), Schulz2005a (2/108 in PXE patients), Schulz2006, Fabre2005, Hu2003c, Ringpfeil2001, Shi2007 (3/32), Schulz2005b (2/108), RamsayM 2009 (6/95 PXE alleles, 0.063	
c.3413G>A	p.R1138Q	M	m		E24	ICL8	+	in mixed South African pop.)	278
c.3413G>C	p.R1138P	M	m	16256943	E24	ICL8		LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007, Hu2003c	279
c.3415G>A	p.A1139T	M	m	16256941	E24	ICL8	-	Pfendner 2007, in some cases in combination with c.2070+5 or c.4335delG	280

								Pfendner 2007; Plomp 2004; Poulkheri 2001; Bergen 2000; Chassaing 2004; Gheduzzi 2004 (36/98); Hu 2003b (22/118); Hu 2004 (35/152); LeSaux 2000; Miksch 2005 (45/170); Ringpfeil 2000 (4/16); Ringpfeil 2001b; Schulz 2005a (28/108 in PXE patients, 9/46 in PXE relatives); Struk 2000; Gotting 2004 (33/128 PXE patients; 1/1820 healthy donors); Klein 2001, LeSaux 2001 (18.8% in EU, 4.1% in US), Uitto 2001, Germain2000, Hendig2005 (38/260 in PXE+related, 1/200 in healthy donors); Trip2002(22/2996, all in heterozygous form), Cai2001, Chassaing2005, Bergen2007, LeSaux2002 (2/34 in Afrikaner, 2/14 in other SA), Audo2007, Ringpfeil2006, Hu2003a, Schulz2006, Hu2003c, Ringpfeil2001, Bowen2007, Shi2007 (5/32), Schulz2005b (28/108 in PXE, 9/46 in relatives), Drera2007 (experimentally validated: in trans with G1133C), Faria 2013, RamsayM 2009 (9/95 PXE alleles, 0.095 in	
				16256935					
c.3421C>T	p.R1141X	M	n		E24	ICL8	+	mixed South African pop.), LaRusso 2010 (32/140), Nitschke 2012	281
c.3427C>T	p.Q1143X	M	n	16256929	E24	ICL8	-	Pfendner 2007	282
				16256866				Chassaing 2004; Meloni 2001; Miksch 2005 (9/170); Ringpfeil 2001b; Struk 2000, Pfendner 2007, Uitto2001, LeSaux2001, Chassaing2005,	
c.3490C>T	p.R1164X	M	n		E24	ICL8	+	Bergen2007, Wang2001, Hu2003c, Ringpfeil2001, Shi2007 (6/32)	283
c.3491G>A	p.R1164Q	M	m	16256865	E24	ICL8	+	Pfendner 2007; Miksch 2005 (1/170), Ringpfeil2006	284
c.3505_3506+2delAG GT		M	i/e, pot. splice	16256851_16256848	E24-I24	ICL8_		Schulz2006	285
c.3506+15G>A		?	-	16256835	I24		-	Schulz2005a (1/266 in AAA patiens, 0 in PXE patients)	286
c.3506+83C>A		P	-	16256767	I24			Meloni2001, HapMap, Bowen2007	287
c.3506+172C>T		P	-	16256678	I24		-	HapMap	288
				16255437				Miksch2005, HapMap, LeSaux2002 (1/48), RamsayM 2009 (Found in 2/10 black healthy, 3/24 PXE patient in south african pop, do not	
c.3507-16T>C		P	-		I24			mention if heteroz. or homoz.)	289
				16255424				Miksch2005(P), LeSaux2002 (P;1/48), Audo2007 (M, splice acceptor	
c.3507-3C>T		P/M	i, pot. splice		I24		-	site), Schulz2006 (M, splicing)	290
c.3507-1G>A		M	i, pot. splice	16255422	I24		-	Miksch 2005 (2/170)	291
c.3544dupC	p.L1182PfsX1278	M	dup, fs	16255384	E25	TMH16_		Chassaing 2005, Bergen2007, Gheduzzi2004 (1/98)	292
c.3608G>A	p.G1203D	M	m	16255320	E25	TMH17	-	LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007, Hu2003c	293
c.3633+55T>C		P	i	16255240	I25			LeSaux2002 (8/48), HapMap	294
c.3633+90G>A		P	i	16255205	I25		-	LeSaux2000, LeSaux2002 (21/48)	295
c.3633+529A>G		P	i	16254766	I25			HapMap	296
c.3634-506G>A		P	i	16253946	I25		-	HapMap	297
c.3634-394C>T		P	i	16253834	I25		-	HapMap	298
c.3634-130G>A		P	i	16253570	I25		-	HapMap	299
c.3634-3C>A		M	i, pot. splice	16253443	I25			Chassaing2004, Chassaing2005, Bergen2007	300
				16253413				Hu 2003; Hu 2004; Miksch 2005 (2/170); Noji 2004; Pfendner 2007,	
c.3661C>T	p.R1221C	M	m		E26	ICL9	+	Bergen2007, Costrop 2010	301
c.3662G>A	p.R1221H	M	m	16253412	E26	ICL9	+	Cotton 1998, Pfendner 2007, Nitschke 2012	302
c.3668G>A	p.W1223X	M	n	16253406	E26	ICL9	-	Chassaing2004, Chassaing2005, Bergen2007	303
c.3676C>A	p.L1226I	M	m	16253398	E26	ICL9		Pfendner 2007	304
c.3701A>T	p.E1234V	M?	m	16253373	E26	ICL9		MartinL 2008 (found in a carrier, who has symptome, that closely mimic PXE,no controlls, does not describe the proband in the paper)	305
c3703C>T	p.R1235W	M	m	16253371	E26	ICL9	+	Miksch 2005 (2/170)	306
c.3709C>T	p.Q1237X	M	n	16253365	E26	ICL9	-	LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007, Hu2003c	307
				16253362				Chassaing 2004, Chassaing2005, Bergen2007, MartinL 2008 (in heterozygotes	
c3712G>C	p.D1238H	M	m		E26	ICL9		with symptomes, that closely mimic PXE)	308
c.3715T>C	p.Y1239H	M	m	16253359	E26	ICL9		Schulz2006, Schulz2005b (1/108)	309
c.3722G>A	p.W1241X	M	n	16253352	E26	ICL9	-	Pfendner 2007	310
				16253351				LeSaux2001 (although called a "neutral variant" this nucleotide change is only present in 1/244 alleles and in none of the control chromosomes), Schulz2006	
c.3723G>C	p.W1241C	P/M	m		E26	ICL9		(M), Schulz2005b (P; 1/108 in PXE, 1/46 in relatives)	311
c.3735G>T	p.E1245D	M	m, pot.splice	16253339	E26	ICL9		Miksch 2005 (1/170)	312
c.3735G>A	p.E1245E	M	s, pot. splice	16253339	E26	ICL9	-	Miksch 2005 (1/170)	313
c.3735+643A>G		P	i	16252696	I26			HapMap	314
c.3736-716G>T		P	i	16252382	I26			HapMap	315
c.3736-502C>T		P	i	16252168	I26		+	HapMap	316
c.3736-334A>C		P	i	16252000	I26			HapMap	317
c.3736-233A>C		P	i	16251899	I26			HapMap	318
				16251667				Miksch 2005 (1/170), Uitto2001, LeSaux2001 (1/116 in EU), Ringpfeil2001b, Bergen2007, Gheduzzi2004 (3/98), Ringpfeil2000 (1/16), Hu2004 (1/152), Schulz2006, Hu2003c, Ringpfeil2001,	
c.3736-1G>A		M	i, pot. splice		I26		-	Schulz2005b (1/108), Nitschke 2012	319
c.3769_3770insC	p.L1259fs	M	ins, fs	16251633_16251632	E27	ICL9		Miksch 2005 (2/170)	320
c.3774_3775insC	p.W1259Lfs	M?	ins, fs	16251628_16251627	E27	ICL9		Pfendner 2007, Sato et al 2009 (associates with angioid streaks, in a study collecting patients with AS but not with the full diagnosys of PXE, 1/108 AS allele and 0/294 control allele)	321
				16251627				LeSaux2000 (causes X at 3815-3817), Uitto2001, LeSaux2001 (1.7% in EU, 0 in US), Bergen2000, Hu2003b (8/118), Miksch2005 (2/170), Chassaing2005, Bergen2007, LeSaux2002 (1/14), Hu2004 (11/152), Hu2003a, Schulz2006, Hu2003c, Ringpfeil2001, Schulz2005b (1/108), RamsayM 2009 (1/47 alleles in mixed South African PXE pop.), Plomp	
c.3775delT	p.W1259Gfs	M	del, fs		E27	ICL9		2009	322
c.3787G>A	p.G1263R	M	m	16251615	E27	ICL9	+	Vanakker2008 (1/87)	323
c.3798delT		M	fs	16251604	E27	ICL9		Bergen2007	324
				16251599				LeSaux2001 (23/244 of PXE chromosomes, 31/200 control chromosomes), HapMap, Miksch2005 (no segregation with disease+in LD with other PXE mut), LeSaux2002 (4/48 - no segregation with disease), Morcher2003, Wang2001 (0.04 in Africans; 0.06 in Chinese; 0.14 in Oji-Cree; 0.16 in South Asians; 0.17 in Inuit; 0.30 in Caucasians), Ringpfeil2000 (listed as mut.; 3/16), Gheduzzi2004, Hu2003a (G:0.3; A:0.7), Schulz2006, Germain2000 (24/124), Meyer2005, Schulz2005b (15/108 in PXE, 14/46 in relatives), SatoN2009 (found in healthy (48/300 control alleles) and AS (34/108 AS alleles) individuals in a study collecting patients with AS but not with the full diagnosys of PXE), QiaoliLi2011 (in association with the	
c.3803G>A	p.R1268Q	P	m		E27	ICL9	+	development of AS)	325
c.3818G>A	p.R1273K	M	m	16251582	E27	ICL9	-	Meyer2005	326
c.3821_3868del48	p.1274Ydel16	M	del	16251581_15251534	E27	ICL9		Hu2004 (1/152)	327
c.3823C>T	p.R1275X	M	n	16251579	E27	ICL9	+	Chassaing 2005; Gheduzzi 2004 (3/98), Bergen2007	328
c.3871G>A	p.A1291T	?	m	16251531	E27	ICL9	+	Hefferon2007	329
c.3877G>A	p.E1293K	M	m	16251525	E27	ICL9	-	Meyer2005	330
c.3880_3882del	p.K1294del	M ?	del, pot. splic	16251522_16251520	E27	ICL9		Pfendner 2007 (no control alleles were investigated)	331
c.3882+531C>T		P	i	16250989	I27		-	HapMap	332
c.3882+922G>C		P	i	16250598	I27			HapMap	333
c.3883-46A>G		P	i	16248934	I27			LeSaux2000	334
c.3883-24G>A		P	i	16248912	I27		-	Miksch2005	335
c.3883-6G>A	p.A1295fsX1321 qain of ss	M/P	i, pot. splice	16248894	I27		+	Miksch 2005 (1/170) gain of ss mutation which causes frame shift and premature stop; LeSaux2002 (only reported as a polymorphism, but 1/48 PXE alleles and they have no controls!), Schulz2006 (M, altered splicing)	336
c.3883-5G>A		M/P	i, pot. splice	16248893	I27		-	Pfendner 2007 (no control alleles were investigated)	337
c.3892G>T	p.V1298F	M	m	16248879	E28	ICL9 (WalkerA)		Pfendner 2007; LeSaux 2001 (0 in EU, 2.7% in US), Chassaing2005, Bergen2007, Hu2003c	338
c3895G>A	p.G1299S	M	m	16248876	E28	ICL9 (WalkerA)	-	Chassaing 2005, Bergen2007	339
c.3902C>T	p.T1301I	M	m	16248869	E28	ICL9 (WalkerA)	-	LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007, Schulz2005a (1/108 in PXE), Schulz2006, Hu2003c, Schulz2005b (1/108)	340
c.3904G>A	p.G1302R	M	m	16248867	E28	ICL9 (WalkerA)	+	Pfendner 2007; Hu 2003c; Hu 2004 (1/152); LeSaux 2001 (0 in EU, 5.4% in US); Miksch 2005 (1/170), Chassaing2005, Bergen2007, Shi2007 (3/32), Ramsay M2009	341
c.3907G>C	p.A1303P	M	m	16248864	E28	ICL9 (WalkerA)		Pfendner 2007; Hu 2003c; Hu 2004 (1/152); LeSaux 2001 (2.6% in EU, 1.4% in US); Miksch 2005 (1/170), Chassaing2005, Bergen2007	342
c.3912delG	p.S1305fsX1358	M	del, fs	16248859	E28	ICL9		Miksch 2005 (1/170)	343

c.3919T>C	p.S1307P	M	m	16248858	E28	ICL9 (WalkerA)		Chassaing 2005; Bergen2007	344
c.3932G>A	p.G1311E	M	m	16248839	E28	ICL9	-	Schulz2005a (?/108 in PXE; 0/400 in controls), Schulz2006, Schulz2005b (1/108)	345
c.3940C>T	p.R1314W	M	m	16248831	E28	ICL9	+	LeSaux 2000 (located at WalkerA in NBF2); LeSaux 2001 (0 in EU, 4.1% in US); Schulz 2005a (1/108 in PXE); Pfendner 2007, Uitto2001, Chassaing2005, Bergen2007, Schulz2006, Hu2003c, Ringpfeil2001, Schulz2005b (1/108), in LD with 4042-30C>T? (Shi2007), Nitschke 2012	346
c.3941G>A	p.R1314Q	M	m	16248830	E28	ICL9	+	Pfendner 2007; Klein 2001, LeSaux 2001 (1/116 in EU); Miksch 2005 (1/170), Chassaing2005, Bergen2007, Schulz2005a (1/108 in PXE, 1/46 in PXE relatives), Schulz2006, Hu2003c, Schulz2005b (1/108 in PXE, 1/46 in relatives)	347
c.3961G>A	p.G1321S	M	m	16248810	E28	ICL9	-	LeSaux 2001 (1/74 in US), Chassaing2005, Bergen2007, Hu2003c	348
c.3978C>T	p.D1326D	P	s	16248793	E28	ICL9	+	Wang2001 (0.03 in Caucasians)	349
c.4004T>C	p.L1335P	M	m	16248767	E28	ICL9		Chassaing 2004, Chassaing2005, RamsayM 2009 (2/48 PXE allles, 0.063 in mixed South African pop.)	350
c.4004T>A	p.L1335Q	M	m	16248767	E28	ICL9		Pfendner 2007	351
c.4015C>T	p.R1339C	M	m	16248756	E28	ICL9	+	Pfendner 2007; Gheduzzi 2004 (3/98); LeSaux 2001 (0 in EU, 2.7 in US); Miksch 2005 (11/170); Struk 2000, Uitto2001, Chassaing2005, Bergen2007, LeSaux 2002 (18/34 in Afrikaner, 0 in other SA), Hu2003c, Ringpfeil2001, Shi2007 (2/32), Faria 2013, RamsayM 2009 (39/95 PXE alleles, 0.411 in mixed South African pop.), LaRUsso2010 (11.5% in mediterraneas, 9.1% of scandinavians of mutated alleles)	352
c.4016G>A	p.R1339H	M	m	16248755	E28	ICL9	+	Pfendner 2007;	353
c.4016G>T	p.R1339L	M	m	16248755	E28	ICL9		Miksch 2005 (1/170)	354
c.4025T>C	p.I1342T	M	m	16248746	E28	ICL9		Pfendner 2007	355
c.4036C>T	p.P1346S	M	m	16248735	E28	ICL9	-	Chassaing 2005; Gheduzzi 2004 (1/98), Bergen2007	356
c.4041+49C>T		P	i	16248681	I28		+	Miksch2005	357
c.4041G>C	p.Q1347H	M	m, pot.splice	16248730	E28	ICL9 (Q-loop)		LeSaux 2001 (0 in EU, 2.7% in US, probably also a splice site mutation), Pfendner 2007, Chassaing2005, Bergen2007, Hu2003c, may also result in aberrant splicing, a similar mutation in ABCC7 does, although both correct+mutant splice variants are expressed due to the use of a cryptic splice site (LeSaux2001)	358
c.4042-30C>T		P	i	16248681	I28		+	LeSaux2000, HapMap, Miksch2005, LeSaux2002 (18/48), Schulz2005a (?), Bowen2007, Shi2007 (3/32), in LD with 3940C>T? (Shi2007)	359
c.4048A>C	p.I1350L	P ?	m	16248645	E29	ICL9		LeSaux2002 (1/48 PXE patients - no controls, no evidence for it to be polymorphism!)	360
c.4060G>C	p.G1354R	M	m	16248633	E29	ICL9		Pulkkinen2001, Chassaing2005, Uitto2001, Bergen2007, Hu2003c	361
c.4069C>T	p.R1357W	M	m	16248624	E29	ICL9	+	Miksch 2005 (1/170), Noji2004	362
c.4081G>A	p.D1361N	M	m	16248612	E29	ICL9	+	LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007, Hu2003c	363
c.4104delC	p.D1368Efs	M	del, fs	16248589	E29	ICL9		LeSaux2001 (0.9% in EU, 0 in US), Chassaing2005, Bergen2007, LeSaux2002 (1/14), RamsayM 2009 (1/47 alleles in mixed South African PXE pop.)	364
c.4182G>T	p.K1394N	M	m	16248511	E29	ICL9		Hu2003b (1/118), Chassaing2005, Bergen2007, Hu2004 (1/152)	365
c.4182delG	p.N1394fsX8	M	del, fs	16248511	E29	ICL9		Hu2003b (4/118), Chassaing2005, Bergen2007, Hu2004 (2/152), Schulz2005a (3/108 in PXE, 0/400 in controls), Schulz2006, Schulz2005b (3/108), Vanakker2008	366
c.4192C>T	p.R1398X	M	n	16248501	E29	ICL9	+	LeSaux 2001 (1/116 in EU), Pfendner 2007, Chassaing2005,	367
c.4198G>A	p.E1400K	M	m	16248495	E29	ICL9	+	Chassaing 2004, Chassaing2005, Gheduzzi2004 (4/98), Bergen2007, Faria 2013,	368
c.4208+9G>A		P	i	16248476	I29		+	Miksch2005, Schulz2005a (5/266 in AAA)	369
c.4208+1014T>A		P	i	16247471	I29			HapMap	370
c.4209-1100C>T		P	i	16245729	I29		+	HapMap	371
c.4209C>A	p.S1403R	M	m, pot.splice	16244629	E30	ICL9 (Signature)		Hendig 2005 (1/260) may also result in aberrant splicing since it is the first position of exon 30, but Hending's paper does not mention it; Schulz2005a (1/108 in PXE, 0/556 in controls), Schulz2006	372
c.4213G>A	p.G1405S	M	m	16244625	E30	ICL9 (Signature)	-	Vanakker2008 (1/76)	373
c.4220insAGAA		M	ins, fs	16244618	E30	ICL9		Uitto2001, LeSaux2001, Bergen2000, Hu2003b (1/118), Chassaing2005, Bergen2007, Hu2004 (1/152), Plomp2004, Hu2003c, Ringpfeil2001	374
c.4253G>A	p.R1418Q	M	m	16244585	E30	ICL9	+	Meyer2005	375
c.4254G>A	p.R1418R	P	s	16244584	E30	ICL9	-	Schulz2005a (6/266 in AAA; 2/244 in controls)	376
c.4271T>C	p.I1424T	M	m	16244567	E30	ICL9		LeSaux 2001 (1/74 in US), Chassaing2005, Bergen2007, Hu2003c	377
c.4279G>A	p.E1427K	M?	m	16244559	E30	ICL9 (WalkerB)	+	Sato et al 2009 (associates with angioid streaks, in a study collecting patients with AS but not with the full diagnosys of PXE, 1/108 AS allele and 0/300 control allele)	378
c.4305C>T	p.G1435G	P	s	16244533	E30	ICL9	-	Miksch2005	379
c.4306_4312del	p.T1436fs	M	fs	16244532_16244526	E30	ICL9_		Plomp 2008 (o/140 control chromosomes)	380
c.4318delA	p.1440fsX1464	M	del, fs	16244520	E30	ICL9		Chassaing 2005; Gheduzzi 2004 (2/98), Bergen2007	381
c.4403+11C>G		P	i	16244424	I30			Hendig2005 (2/260), Schulz2005a (2/108 in PXE, 0 in controls)	382
c.4404-81C>T		P	i	16244179	I30		+	Hendig (1/260)	383
c.4404-76A>G		P	i	16244174	I30			Chassaing2004, HapMap, Hendig2005, Gheduzzi2004, Schulz2006	384
c.4404-31G>A		P	i	16244129	I30		-	Hendig2005, HapMap, Miksch2005, Gheduzzi2004, Schulz2006	385
c.4434delA	p.R1479fsX25	M	del, fs	16244068	E31	ICL9		Hendig 2005 (2/260) alters the aa. sequence of the C-terminus and results in the loss of the regular termination codon; Schulz2006	386
c.4441G>A	p.G1481S	M	m	16244061	E31	ICL9	+	Pfendner 2007	387
c.4448C>T	p.P1483L	?	m	16244054	E31	ICL9	+	Hefferon2007	388
c.4501G>A	p.G1501S	M	m	16244001	E31	ICL9	-	Pfendner 2007	389
c.4512+17G>A		P	i	16243973	3'UTR		+	LeSaux2000, Miksch2005, LeSaux2002 (2/48)	390
c.4512+38G>A		P	i	16243952	3'UTR		+	Miksch2005	391
c.1-2787del	p.M1_R929 ! uncorrect	M	del	16317291_16267141	E01-21			exon 21	392
c2996_4208del	p.I1000_S1403del;W1404fsX1463 p.1000Wfs...	M	del	16259790_16288485				LeSaux 2001 (4.3% in EU, 28.4% in US); Ringpfeil 2001; Miksch 2005 (19/170)indicated as in frame large del but out of frame deletion, Shi2007 (2/32), Nitschke 2012	393
	A995del405	M	del		E23-29			Hu2003b (14/118), Hu2004 (17/152)	394
c.2997_4208del		M	del	16259789_16248485				Katona2005, Bergen2007, Audo2007, Schulz2005a (5/108 in PXE, 3/46 in PXE relatives), Ringpfeil2006, Schulz2006, Hu2003c, Ringpfeil2001, SatoN 2009 (del_exon23), RamsayM 2009 (del 23-29: 3/95 PXE alleles, 0.043 in mixed South African pop.), LaRusso2010 (18/140)	395
del ABCC6 ABCC1	p.M1-V1503del	M	del		E01-31			Ringpfeil2001b, Hu2003a	396
MYH11,+ 5 genes	p.M1-V1503del	M	del		E01-31			Miksch 2005 (1/170)large deletion	397
MYH11,+ 7 genes	p.M1-V1503del	M	del		E01-31			Miksch 2005 (1/170)large deletion	398
delABCC6		M	del		delABCC6			Miksch 2005 (1/170)large deletion	399
c.(?_-37)_(*568_?)del		M	del					LeSaux2001, Bergen2000, Hu2003b, Bergen2007, Hu2004 (2/152), Hu2003c	400
del ABCC6 ABCC1 MYH11		M	del					Costrop 2010	401
exon24_25		M	del		E24-25			Hu2003b, Meloni2001, Hu2003c	402
c.1-3189_36+1181del		M	del	1632048_16316075	5'UTR-I1			Katona2005	403
c.37-?_219+?del		M	del	16315688-?_16315506+?del	E2del			Chassaing2007	404
c.2667-42_2787+1623del		M	del	16267303_16265518	I20-I21			Costrop 2010	405
c.999-?_1176+?del		M	del	16296035-?_16295858+?del	E9del			Chassaing2007	406
c.1091_1338+3149del		M	del	16295943_16290255	E9-I10			Costrop 2010	407
c.1780-?_1867+?del		M	del	16281068-?_16280981+?del	E14del			Chassaing2007	408
ABCC6 del15 (1867+1530_1943+949del)	fs,p.624X	M	del		E15			Costrop 2010	409
c.3307-904_3506+660del		M	del	c.3307-904_3506+660del	E24del			LeSaux2001, Chassaing2005, Bergen2007, LeSaux2002 (2/14), Hu2003c	410
c.3307-1006_3882+1582del		M	del	16256043_16253102del	E24-E27del			Costrop 2010	411
c.4209-?_4403+?del		M	del	16244629-?_16244435+?del	E30del			Costrop 2010	412
c.4209-?_4512+?del		M	del	16244629-?_16243990+?del	E30-E31			Costrop 2010	413
								Costrop 2010	414

Supp.Table 2

A	B	C	D	E	F	G	H	I	
sequence variation at DNA level	sequence variation at protein level	Mutation/ Polym.	type of mut. (exonic intronic)	genomic pos. according to ENSG00000091262.10; 2013. July	genomic region affected	protein region affected	C>T at 5'CpG	references (allele frequency data and comments)	
c.37-1G>A		M	i, pot. splice	16315689	I1		-	Schulz2006	1
c.105delA	p.S37fsX80	M	del, fs	16315620	E2	TMH1-TMH2		Miksch 2005 (1/170)	2
c.113G>C	p.W38S	M	m	16315612	E2	TMH1		Schulz2006	3
c.175_179del	p.G58fs	M	del, fs	16315550_16315546	E2	ICL1		Vanakker (3/76)	4
c.179_187del	p.R60_Y62del	M	del	16315546_16315538	E2	ICL1		Pulkkinen 2001;Miksch 2005 (1/170) in frame del; Chassaing2005, Bergen2007, Hu2003c	5
c.179_195del		M	del, fs	16315546_16315530	E2	ICL1		LeSaux2001 (0.9% in EU, 0 in US), Chassaing2005, Bergen2007, Hu2003c	6
c.220_222del	p.V74del	M	del	16313804_16313802	E3	TMH2		Chassaing 2004 also a splice mutation since this is the first codon of exon 3, Chassaing2005, Bergen2007	7
c.373G>T	p.E125X	M	n	16313512	E4	ICL2_		Miksch 2005 (1\170)	8
c.373G>A	p.E125K	M	m	16313512	E4	ICL2	+	Vanakker2008 (1/76)	9
c.386G>A	p.G129E	M	m	16313499	E4	ICL2	-	Miksch 2005 (1\170)	10
c.450_451insC	p.A151Rfs	M	ins, fs	16313435_16313434	E4	ECL3_		Nitschke 2012	11
c.496C>T	p.R166C	M	m	16308285	E5	ECL3	+	MartinL 2008 (found in a carrier, who has symptome, that closely mimic PXE, also reports on a PXE patient having c.496C>T, in compound heteroz. state with c.ABCC6del, but do not describes the patient in this paper, no controlls)	12
c.595C>T	p.Q199X	M	n	16308186	E5	ICL3	-	Yoshida2005, Chassaing2005	13
c.676G>A	p.G226R	M	m	16302703	E7	ICL3	-	Chassaing2005, Bergen2007	14
c.681C>G	p.Y227X	M	n	16302698	E7	ICL3		Chassaing 2005, Meloni2001, Bergen2007	15
c.708_709dupCT	p.W237Sfs*21	M	dup	16302671_16302670	ICL3	ICL3		Tan2012 (case report, homozygous mutation in this patient, born from consanguineous parents, no controls)	16
c.724G>T	p.E242X	M	n	16302655	E7	ICL3		Pfendner 2007 (1/542)	17
c.754C>T	p.L252F	M	m	16302625	E7	ICL3	-	Schulz2006 (not found in 200 control alleles, relatively conserved position, but present together with 2 further mutations in the PXE patient!)	18
c.865_889del		M	del, fs	16297400_16297376	E8	ICL3		LeSaux2002 (found in a heterozygous state in an apparently healty (control) person)	19
c.938_939insT		M	ins, fs	16297327_16297326	E8	TMH6		LeSaux2001 (1/202), Chassaing2005, Bergen2007, LeSaux2002 (1/34 in Afrikaner, 1/14 in other SA), Hu2003c	20
c.951C>A	p.S317R	M	m	16387314	E8	TMH6		Miksch 2005 (1/170)	21
c.951C>G	p.S317R	M	m	16297314	E8	TMH6		Pfendner 2007 (2/542);	22
c.960delC	p.I320fs	M	del, fs	16297305	E8	TMH6		Chassaing 2005; Meloni 2001, Bergen2007, Hu2003c	23
c.998+2_998+3delTG		M	i, pot. splice	16297264_16297265	I8			Chassaing2005, Bergen2007	24
c.1064T>G	p.L355R	M	m	16295970	E9	TMH7		Miksch 2005 (1/170), Nitschke2012	25
c.1087C>T	p.Q363X	M	n	16295947	E9	TMH7	-	Pfendner 2007 (1/541)	26
c.1088_1120del	p.363_374QTLF EQQNMYRdel	M	del	16295946_16295914	E9	TMH7-ICL4		Chassaing 2004 in frame deletion, Chassaing2005, Bergen2007, MartinL 2008	27
c.1091C>G	p.T364R	M	m	16295943	E9	TMH7		Pulkkinen 2001; Gheduzzi 2004 (1/98); Hu 2003; Miksch 2005 (1/170), Chassaing2005, Bergen2007, Hu2003c	28
c.1091C>T	p.T364M	M	m	16295943	E9	TMH7	+	Vanakker2008	29
c.1108A>G	p.N370D	M	m	16295926	E9	TMH7		Miksch 2005 (2/170)	30
c.1132C>T	p.Q378X	M	n	16295902	E9	ICL4	-	Pfendner 2007 (4/542); Cai 2001 (possibly in LD with 1077A>G; 1141T>C); Gheduzzi 2004 (5/98); Miksch 2005 (6/170); Chassaing2005, Bergen2007, Schulz2006, Hu2003c, Hu2003b, Schulz2005b, LaRusso2010 (3.8% of mediterraneans, 9.1% of scandinavianmutant alleles)	31
c.1144C>T	p.R382W	M	m	16295890	E9	ICL4	+	Pfendner 2007 (2/542)	32
c.1171A>G	p.R391G	M	m	16295863	E9	ICL4		Pfendner 2007 (3/542); Pulkkinen 2001; Chassaing 2004; Miksch 2005 (2/170); Gotting 2004, Chassaing2005, Bergen2007, Ringpfeil2006, Schulz2006, Nitschke2012, Costrop2010	33
c.1176G>C	p.K392N	M	m	16295858	E9	ICL4		Pfendner 2007 (1/542), also a splice mutation since this position is within the last codon of exon 9	34
c.1192A>G	p.S398G	M	m	16292024	E10	ICL4		Miksch 2005 (1/170)	35
c.1194C>G	p.S398R	M	m	16292022	E10	ICL4		Vanakker2008 (1/76)	36
c.1233T>G	p.N411K	M	m	16291983	E10	ICL4		LeSaux 2001 (0 in EU, 1.4% in US), Chassaing2005, Bergen2007, Gheduzzi2004 (1/98), Hu2003c	37
c.1244T>C	p.V415A	M	m	16291972	E10	ICL4		Schulz2006 (not found in 200 control alleles, relatively conserved position, but present together with 2 further mutations in the PXE patient!)	38
c.1318T>G	p.C440G	M	m	16291898	E10	TMH8		Gheduzzi 2004 (1/98); Chassaing 2005, Bergen2007	39
c.1363G>C	p.A455P	M	m	16286755	E11	TMH9		Uitto 2001, LeSaux2001, Chassaing2005, Hu2003c, Ringpfeil2001	40
c.1388T>A	p.L463H	M	m	16286730	E11	TMH9		Pfendner 2007 (1/542)	41
c.1396A>T	p.N466Y	M	m	16286722	E11	TMH9		Vanakker2008 (1/76)	42
c.1460G>A	p.R487Q	M	m	16284196	E12	ICL5	+	Schulz2006	43
c.1484T>A	p.L495H	M	m	16284172	E12	ICL5		Pfendner 2007 (1/542); Miksch 2005 (1/170)	44
c.1491C>A	p.N497K	M	m	16284165	E12	ICL5		Schulz2006	45
c.1498A>C	p.T500P	M	m	16284158	E12	ICL5		Vanakker2008 (1/76)	46
c.1505A>T	p.K502M	M	m	16284151	E12	ICL5		Chassaing 2005, Bergen2007	47
c.1552C>T	p.R518X	M	n	16284104	E12	ICL5	+	Pfendner 2007 (2/542); Chassaing 2004; Gheduzzi 2004 (11/98); Meloni 2001; Miksch 2005 (1/170); Chassaing2005, Bergen2007, Ringpfeil2006, Schulz2006, Hu2003c, Hu2003b, Nitschke2012, Costrop 2010	48
c.1553G>A	p.R518Q	M	m	16284103	E12	ICL5	+	Pfendner 2007 (4/542); Chassaing 2004; Gheduzzi 2004 (5/98); LeSaux 2000; Miksch 2005 (4/170); Ringpfeil 2001, LeSaux 2001 (0.9% in EU, 1.4% in US); Uitto 2001, Chassaing2005, Bergen2007, LeSaux2002 (1/14), Hu2003c, Faria 2013, RamsayM 2009 (3/95 PXE alleles, 0.032 in mixed South African pop.), Costrop 2010	49
c.1563G>C	p.E521D	M	m	16284093	E12	ICL5		Vanakker2008 (1/76)	50
c.1574_1575insG	p.L525fsX73	M	ins, fs	16284082_16284081	E12	ICL5		Schulz2006	51
c.1603T>C	p.S535P	M	m	16284053	E12	TMH10		Pfendner 2007 (1/542)	52
c.1652T>C	p.F551S	M	m	16282815	E13	TMH10		Miksch 2005 (1/170)	53
c.1674delC	p.A558fs	M	del, fs	16282793	E13	ECL6_		Vanakker2008 (1/76)	54
c.1685T>C	p.M562T	M	m	16282764	E13	ECL6		Plomp 2008 (0/140 control chromosome)	55
c.1703T>C	p.F568S	M	m	16282698	E13	ICL6		LeSaux 2001 (0 in EU, 1.4% in US), Pfendner 2007 (1/542), Chassaing2005, Bergen2007, Ringpfeil2006, Hu2003c	56
c.1769C>T	p.S590F	M	m	16281097	E13	TMH11	-	Nitschke2012	57
c.1780-29T>A		M	i, pot. splice	16281067	I13			Chassaing 2005, Bergen2007	58
c.1781C>T	p.A594V	M	m	16281050	E14	TMH11	-	Miksch 2005 (2/170) c.1781 is the 2nd nucleotide position of e14 - possible splice mut?	59
c.1798C>T	p.R600C	M	m	16280991_16280990	E14	ICL6	+	Pfendner 2007 (1/542), Costrop 2010	60
c.1857_1858insC		M	fs	16278896	E14	ICL6_		Pfendner 2007	61
c.1868-5T>G		M	i, pot. splice	16276787_16276766	I14			Chassaing 2005, Bergen2007	62
c.1944_1965del	p648fsX688	M	del, fs	16276764_16276742	E16	ICL6 (WalkerA)		LeSaux2001, Hu2003b (2/118), Bergen2000, Hu2004 (2/152), Hu2003a, Hu2003c, Ringpfeil2001	63
c.1967_1989del		M	fs	16276744	E16	ICL6_		Bergen 2000	64
c.1987G>T	p.G663C	M	m	16276736	E16	ICL6 (WalkerA)		Pfendner 2007 (1/542)	65
c.1995delG	p.A667fsX20	M	del, fs	16276713	E16	ICL6 (WalkerA)		LeSaux2001 (0.9% in EU, 0 in US), Hendig2005 (3/152), Chassaing2005, Bergen2007, Schulz2005a (3/108 in PXE patients), Schulz2006, Hu2003c, Schulz2005b	66
c.2018T>C	p.L673P	M	m	16276701	E16	ICL6		LeSaux 2001, Chassaing2005, Bergen2007, LeSaux2002 (1/34 in Afrikaner, 0 in other SA), Hu2003c	67
c.2030T>C	p.L677P	M	m	16276423	E17	ICL6 (Q-loop)		Miksch 2005 (1/170)	68
c.2093A>C	p.Q698P	M	m	16276419	E17	ICL6		Pfendner 2007	69
c.2097G>T	p.E699D	M	m	16276354	E17	ICL6	-	Pfendner 2007 (1/542)	70
c.2162G>A	p.W721X	M	n	16276339	E17	ICL6		Miksch 2005 (1\170)	71
c.2177T>C	p.L726P	M	m	16276279_16276278	E17	ICL6		Pfendner 2007 (1/542), RamsayM 2009 1/47 in mixed South African PXE alleles, 0/200 control alleles)	72
c.2237_2238ins10		M	fs	16276271	E17	ICL6_		Pfendner 2007	73
c.2245C>T	p.Q749X	M	n	16272833_16272834	E17	ICL6	-	Chassaing 2005, Bergen2007, Hu2003a	74
c.2248-12_11delTT		M	i, pot. splice	16272823_16272824	I17			Hu2003b (reported as splice site mutation, but no further evidence to support this), Chassaing2005, Bergen2007, Hu2004 (1/152)	75
c.2248-2_2248-1del	p.750_805del	M	i, pot. splice	16272818	I17			Gheduzzi2004 (1/98) - RT-PCR and sequencing show that this mutation results in skipping of exon18	76
c.2252T>A	p.M751K	M	m		E18	ICL6		Pfendner 2007 (1/542); Hendig 2005 (5/152); Gotting 2004, Schulz2005a (2/108 in PXE patients), Schulz2006	77

c.2263G>A	p.G755R	M	m	16272807	E18	ICL6 (Signature)	+	Pfendner 2007 (2/542)	78
c.2278C>T	p.R760W	M	m	16272792	E18	ICL6 (Signature)	+	Pfendner 2007 (3/542); Schulz 2005a (1/108 in PXE patients); Gotting 2004; Miksch 2005 (1/170); Hendig 2005 (5/260), Schulz2006	79
c.2279G>A	p.R760Q	M	m	16272791	E18	ICL6 (Signature)	+	Vanakker2008 (2/76)	80
c.2293C>T	p.R765W	M	m	16272777	E18	ICL6 (Signature)	+	Vanakker2008 (1/76)	81
				16272776				Hendig 2005 (4/260); Hu 2003b (1/118); Hu 2004 (1/152); LeSaux 2002; Miksch 2005 (1/170); Schulz 2005a (3/108 in PXE patients); Gotting 2004, Pfendner 2007 (2/542); LeSaux 2001 (0.9% in EU, 0 in US), Chassaing2005, Bergen2007, Schulz2006, Hu2003c, LeBoulanger 2009 (most probably associated with GACI, uncorrectly reported mutation, most probably	
c.2294G>A	p.R765Q	M	m		E18	ICL6 (Signature)	+	R765Q)	82
c.2297C>A	p.A766D	M	m	16272773	E18	ICL6 (Signature)		Chassaing 2004, Chassaing2005, Bergen2007	83
				16272766				LeSaux 2001;Miksch 2005 (1/170), Chassaing2005, Bergen2007, LeSaux2002 (4/34 in Afrikaner, 0 in other SA), Hu2003c, RamsayM 2009 (8/95 PXE alleles, 0.084 in mixed South African pop.)	
c.2304C>A	p.Y768X	M	n		E18	ICL6			84
c.2322delC	p.774Yfs	M	del, fs	16272748	E18	ICL6_		LeSaux2001 (1/74 in US), Chassaing2005, Bergen2007, Hu2003c	85
c.2329G>A	p.D777N	M	m	16272741	E18	ICL6 (WalkerB)	-	Pfendner 2007 (1/542)	86
c.2342C>T	p.A781V	M	m	16272728	E18	ICL6 (WalkerB)	+	Chassaing 2005, Bergen2007	87
c.2378C>G	p.N793L	M	m	16272691	E18	ICL6		Vanakker2008	88
c.2419C>T	p.R807W	M	m	16271480	E19	ICL6	+	Miksch 2005 (1/170) c.2419 is the 4th nucleotide posotion of e19 - possible splice mut?	89
c.2420G>A	p.R807Q	M	m	16271479	E19	ICL6	+	Miksch 2005 (1/170) c.2420 is the 5th nucleotide posotion of e19 - possible splice mut?, Chassaing2005, Bergen2007	90
c.2428G>A	p.V810M	M	m	16271471	E19	ICL6	+	Chassaing 2005; Gheduzzi 2004 (1/98), Bergen2007	91
c.2432C>T	p.T811M	M	m	16271467	E19	ICL6	+	Pfendner 2007 (1/542); Ringpfeil 2006	92
c.2458G>C	p.A820P	M	m	16271441	E19	ICL6		Chassaing 2005; Ghedzzi 2004 (2/98), Bergen2007	93
c.2477T>C	p.L826P	M	m	16271422	E19	ICL6		Plomp 2008 (0/140 control chromosome)	94
c.2511C>A	p.Y837X	M	n	16271388	E19	ICL6		Hefferon2007	95
c.2524C>T	p.Q842X	M	n	16271375	E19	ICL6	-	Miksch 2005 (1\170)	96
				16271357				Uitto2001, LeSaux2001, Chassaing2005, Bergen2007, Noji2004, Hu2003c, Ringpfeil2001, according to reference genome there is an A at this position, but this is also a HapMap polymorphism, with A being the minor allele only present in yoruba and japanese, SatoN2009 (associates with angioid streaks, in a study collecting patients with AS but not with the full diagnosys of PXE, 27/108 AS allele and 1/300 control allele), La Russo2010(onlz present in asians, japanese)	
c.2542delG	p.2848fs	M	del, fs		E19	ICL6			97
c2552T>C	p.L851P	M	m	16271347	E19	ICL6		Hendig 2005 (2/260); Schulz2006 (has been described as mutation, but occurs together with 2 definate mutations!)	98
c.2643G>T	p.R881S	M	m	16269791	E20	ICL6		Pfendner 2007 (1/542)	99
				16267140				LeSaux2000 (mutates donor site, lowers splice potential score from 72.1 to 53.8), Uitto2001, Miksch 2005 (5/170), LeSaux2001 (3.4% in EU, 4.1% in US), Hendig2005 (11/260), Chassaing2005, Bergen2007, Schulz2006, Hu2003c, Ringpfeil2001, Schulz2005b, Nitschke2012,	
c.2787+1G>T		M	i, pot. splice		I21			Costrop 2010	100
c.2814C>G	p.Y938X	M	n	16263684	E22	ICL6		Pfendner 2007 (1/542)	101
c.2820insC		M	fs	14263678	E22	TMH12_		Pfendner 2007	102
c.2831C>T	p.T944I	M	m	16263667	E22	TMH12	-	Pfendner 2007 (1/542)	103
c.2835 _2850del16	p.P946fsX17	M	del, fs	16263663_16263648	E22	TMH12		Hendig 2005 (1/108) out of frame deletion that results in 17 new codons and premature STOP, Schulz2006	104
c.2848G>A	p.A950T	M	m	16263650	E22	TMH12	+	Pfendner 2007 (1/542)	105
c.2891G>C	p.R964P	M	m	16263607	E22	ECL7		Plomp 2008 (0/140 control chromosome)	106
c.2943G>T	p.Q981H	M	m	16263555	E22	ECL7		Vanakker2008 (1/76)	107
c.2974G>C	p.G992R	M	m	#REF!	E22	ECL7		Chassaing 2005; Schulz 2006; Pfendner 2007, Bergen2007	108
c.3074T>C	p.L1025P	M	m	16259712	E23	ICL7		Vanakker2008 (1/76)	109
c.3088C>T	p.R1030X	M	n	16259698	E23	ICL7	+	LeSaux 2001 (1/202), Gheduzzi 2004 (5/98); Chassaing 2005, Bergen2007, LeSaux2002, Hu2003c	110
c.3105_3107delCTT	p.F1036del	M	del	16259681_16259679	E23	ICL7		Nitschke 2012	111
c.3106_3108delTTT	p.F1036del	M	del	16259680_16259678	E23	ICL7		Miksch 2005 (1/170)in frame del	112
c.3141_3143delTCT	p.F1048del	M	del	16259645_16259643	E23	ICL7		Miksch 2005 (1/170)	113
c.3145T>G	p.S1049A	M	m	16259641	E23	ICL7		Schulz2006 (not present in 200 control alleles, relatively conserved position, but occurs together with 2 further mutations in PXE patient!)	114
c.3168C>A	p.D1056E	M	m	16259618	E23	ICL7		Miksch 2005 (1/170)	115
c.3188T>G	p.L1063R	M	m	16259598	E23	TMH14		Schulz2006	116
c.3207C>A	p.Y1069X	M	n	16259579	E23	TMH14		Plomp 2008	117
c.3307-3_-38del and insAGA		M	i, pot. splice	16257052_16257087	I23			Miksch2005(1/170)	118
c.3340C>T	p.R1114C	M	m	16257016	E24	ICL8	+	Ghedzzi 2004 (1/98); Miksch 2005 (1/170); Schulz 2005a (1/108 in PXE patients); Gotting 2004; Klein 2001, Chassaing2005, Bergen2007, Schulz2006, Schulz2005b (1/108), Nitschke 2012	119
c.3341G>C	p.R1114P	M	m	16257015	E24	ICL8		LeSaux 2001 (1/116 in EU), LeSaux2000 (not present in a control panel of 200 chromosomes), Uitto2001, Chassaing2005, Bergen2007, Audo2007, Hu2003c, Ringpfeil2001	120
c.3341G>A	p.R1114H	M	m	16257015	E24	ICL8	+	Hu2003b (1/118), Götting2004(1/128), Chassaing2005, Bergen2007, Hu2004 (1/152), Schulz2005a (1/108 in PXE patients), Schulz2006, Schulz2005b (1/108)	121
c.3362C>T	p.S1121L	M	m	16256994	E24	ICL8	+	Miksch 2005 (3/170)	122
c.3362C>G	p.S1121W	M	m	16256994	E24	ICL8		LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007, Hu2003c	123
c.3364delT	p.S1122Lfs	M	fs	16256992	E24	ICL8		Vanakker2008 (1/76)	124
				16256967				Chassaing 2004; Gheduzzi 2004 (2/98); Hu 2003; Hu 2004;Miksch 2005; Ringpfeil 2000; Schulz 2005a (2/108 in PXE patients); Gotting 2004 (2/128); Klein 2001, Pfendner 2007, Chassaing2005, Bergen2007, Morcher2003,	
c.3389C>T	p.T1130M	M	m		E24	ICL8	+	Schulz2006, Shi2007 (1/32), Schulz2005b (2/108), Martin L2008	125
c.3397G>T	p.G1133C	M	m	16256959	E24	ICL8		Drera2007 (experimentally validated: in trans with R1141X)	126
c.3398G>C	p.G1133A	M	m	16256958	E24	ICL8		Pfendner 2007	127
c.3412C>T	p.R1138W	M	m	16256944	E24	ICL8	+	Ringpfeil 2000 (1/16); Miksch 2005 (1/170), Uitto2001, LeSaux2001, Chassaing2005, Bergen2007, Ringpfeil2006, Schulz2006, Hu2003c, Ringpfeil2001, Shi2007(2/32), HesseRJ 2010	128
c.3413G>A	p.R1138Q	M	m	16256943	E24	ICL8	+	LeSaux 2001 (0.9% in EU, 1.4% in US); Miksch 2005 (3/170); Ringpfeil 2000 (1/16), LeSaux2000, Uitto2001, Chassaing 2005; Gheduzzi 2004 (1/98); Chassaing 2004, Bergen2007, LeSaux2002 (3/34 in Afrikaner, 0 in other SA), Schulz2005a (2/108 in PXE patients), Schulz2006, Fabre2005, Hu2003c, Ringpfeil2001, Shi2007 (3/32), Schulz2005b (2/108), RamsayM 2009 (6/95 PXE alleles, 0.063 in mixed South African pop.)	129
c.3413G>C	p.R1138P	M	m		E24	ICL8		LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007, Hu2003c	130
c.3415G>A	p.A1139T	M	m	16256941	E24	ICL8	-	Pfendner 2007, in some cases in combination with c.2070+5 or c.4335delG	131
c.3421C>T	p.R1141X	M	n	16256935	E24	ICL8	+	Pfendner 2007; Plomp 2004; Poliakinen 2001; Bergen 2000; Chassaing 2004; Gheduzzi 2004 (36/98); Hu 2003b (22/118); Hu 2004 (35/152); LeSaux 2000; Miksch 2005 (45/170); Ringpfeil 2000 (4/16); Ringpfeil 2001b; Schulz 2005a (28/108 in PXE patients, 9/46 in PXE relatives); Struk 2000; Gotting 2004 (33/128 PXE patients; 1/1820 healthy donors); Klein 2001, LeSaux 2001 (18.8% in EU, 4.1% in US), Uitto 2001, Germain2000, Hendig2005 (38/260 in PXE+related, 1/200 in healthy donors); Trip2002(22/2996, all in heterozygous form), Cai2001, Chassaing2005, Bergen2007, LeSaux2002 (2/34 in Afrikaner, 2/14 in other SA), Audo2007, Ringpfeil2006, Hu2003a, Schulz2006, Hu2003c, Ringpfeil2001, Bowen2007, Shi2007 (5/32), Schulz2005b (28/108 in PXE, 9/46 in relatives), Drera2007 (experimentally validated: in trans with G1133C), Faria 2013, RamsayM 2009 (9/95 PXE alleles, 0.095 in mixed South African pop.), LaRusso 2010 (32/140), Nitschke 2012	132
c.3427C>T	p.Q1143X	M	n	16256929	E24	ICL8	-	Pfendner 2007	133
c.3490C>T	p.R1164X	M	n	16256866	E24	ICL8	+	Chassaing 2004; Meloni 2001; Miksch 2005 (9/170); Ringpfeil 2001b; Struk 2000, Pfendner 2007, Uitto2001, LeSaux2001, Chassaing2005, Bergen2007, Wang2001, Hu2003c, Ringpfeil2001, Shi2007 (6/32)	134
c.3491G>A	p.R1164Q	M	m	16256865	E24	ICL8	+	Pfendner 2007; Miksch 2005 (1/170), Ringpfeil2006	135
c.3505_3506+2delAG GT		M	i/e, pot. splice	16256851_16256848	E24-I24	ICL8_		Schulz2006	136

c.3507-1G>A		M	i, pot. splice	16255422	I24		-	Miksch 2005 (2/170)	137
	p.L1182PfsX127			16255384					
c.3544dupC	8	M	dup, fs		E25	TMH16_		Chassaing 2005, Bergen2007, Gheduzzi2004 (1/98)	138
c.3608G>A	p.G1203D	M	m	16255320	E25	TMH17	-	LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007, Hu2003c	139
c.3634-3C>A		M	i, pot. splice	16253443	I25			Chassaing2004, Chassaing2005, Bergen2007	140
				16253413				Hu 2003; Hu 2004; Miksch 2005 (2/170); Noji 2004; Pfendner 2007,	
c.3661C>T	p.R1221C	M	m		E26	ICL9	+	Bergen2007, Costrop 2010	141
c.3662G>A	p.R1221H	M	m	16253412	E26	ICL9	+	Cotton 1998, Pfendner 2007, Nitschke 2012	142
c.3668G>A	p.W1223X	M	n	16253406	E26	ICL9	-	Chassaing2004, Chassaing2005, Bergen2007	143
c.3676C>A	p.L1226I	M	m	16253398	E26	ICL9		Pfendner 2007	144
c3703C>T	p.R1235W	M	m	16253371	E26	ICL9	+	Miksch 2005 (2/170)	145
c.3709C>T	p.Q1237X	M	n	16253365	E26	ICL9	-	LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007, Hu2003c	146
				16253362				Chassaing 2004, Chassaing2005, Bergen2007, MartinL 2008 (in heterozygotes	
c3712G>C	p.D1238H	M	m		E26	ICL9		with symptomes, that closely mimic PXE)	147
c.3715T>C	p.Y1239H	M	m	16253359	E26	ICL9		Schulz2006, Schulz2005b (1/108)	148
c.3722G>A	p.W1241X	M	n	16253352	E26	ICL9	-	Pfendner 2007	149
c.3735G>T	p.E1245D	M	m, pot.splice	16253339	E26	ICL9		Miksch 2005 (1/170)	150
c.3735G>A	p.E1245E	M	s, pot. splice	16253339	E26	ICL9	-	Miksch 2005 (1/170)	151
								Miksch 2005 (1/170), Uitto2001, LeSaux2001 (1/116 in EU),	
				16251667				Ringpfeil2001b, Bergen2007, Gheduzzi2004 (3/98), Ringpfeil2000	
								(1/16), Hu2004 (1/152), Schulz2006, Hu2003c, Ringpfeil2001,	
c.3736-1G>A		M	i, pot. splice		I26		-	Schulz2005b (1/108), Nitschke 2012	152
c.3769_3770insC	p.L1256fs	M	ins, fs	16251633_16251632	E27	ICL9		Miksch 2005 (2/170)	153
								LeSaux2000 (causes X at 3815-3817), Uitto2001, LeSaux2001 (1.7%	
								in EU, 0 in US), Bergen2000, Hu2003b (8/118), Miksch2005 (2/170),	
								Chassaing2005, Bergen2007, LeSaux2002 (1/14), Hu2004 (11/152),	
				16251627				Hu2003a, Schulz2006, Hu2003c, Ringpfeil2001, Schulz2005b (1/108),	
								RamsayM 2009 (1/47 alleles in mixed South African PXE pop.), Plomp	
c.3775delT	p.W1259Gfs	M	del, fs		E27	ICL9		2009	154
c.3787G>A	p.G1263R	M	m	16251615	E27	ICL9	+	Vanakker2008 (1/87)	155
c.3798delT		M	fs	16251604	E27	ICL9		Bergen2007	156
c.3818G>A	p.R1273K	M	m	16251582	E27	ICL9	-	Meyer2005	157
c.3821_3868del48	p.1274Ydel16	M	del	16251581_16251534	E27	ICL9		Hu2004 (1/152)	158
c.3823C>T	p.R1275X	M	n	16251579	E27	ICL9	+	Chassaing 2005; Gheduzzi 2004 (3/98), Bergen2007	159
c.3877G>A	p.E1293K	M	m	16251525	E27	ICL9	-	Meyer2005	160
				16248879				Pfendner 2007; LeSaux 2001 (0 in EU, 2.7% in US), Chassaing2005,	
c.3892G>T	p.V1298F	M	m		E28	ICL9 (WalkerA)		Bergen2007, Hu2003c	161
c3895G>A	p.G1299S	M	m	16248876	E28	ICL9 (WalkerA)	-	Chassaing 2005, Bergen2007	162
								LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007,	
				16248869				Schulz2005a (1/108 in PXE), Schulz2006, Hu2003c, Schulz2005b	
c.3902C>T	p.T1301I	M	m		E28	ICL9 (WalkerA)	-	(1/108)	163
								Pfendner 2007; Hu 2003c; Hu 2004 (1/152); LeSaux 2001 (0 in EU, 5.4%	
				16248867				in US); Miksch 2005 (1/170), Chassaing2005, Bergen2007, Shi2007 (3/32),	
c.3904G>A	p.G1302R	M	m		E28	ICL9 (WalkerA)	+	Ramsay M2009	164
				16248864				Pfendner 2007; Hu 2003c; Hu 2004 (1/152); LeSaux 2001 (2.6% in EU,	
c.3907G>C	p.A1303P	M	m		E28	ICL9 (WalkerA)		1.4% in US); Miksch 2005 (1/170), Chassaing2005, Bergen2007	165
c.3912delG	p.S1305fsX1358	M	del, fs	16248859	E28	ICL9		Miksch 2005 (1/170)	166
c.3919T>C	p.S1307P	M	m	16248858	E28	ICL9 (WalkerA)		Chassaing 2005; Bergen2007	167
				16248839				Schulz2005a (?/108 in PXE; 0/400 in controls), Schulz2006, Schulz2005b	
c.3932G>A	p.G1311E	M	m		E28	ICL9	-	(1/108)	168
								LeSaux 2000 (located at WalkerA in NBF2); LeSaux 2001 (0 in EU, 4.1% in	
								US); Schulz 2005a (1/108 in PXE); Pfendner 2007, Uitto2001,	
				16248831				Chassaing2005, Bergen2007, Schulz2006, Hu2003c, Ringpfeil2001,	
								Schulz2005b (1/108), in LD with 4042-30C>T? (Shi2007), Nitschke	
c.3940C>T	p.R1314W	M	m		E28	ICL9	+	2012	169
								Pfendner 2007; Klein 2001, LeSaux 2001 (1/116 in EU); Miksch 2005	
				16248830				(1/170), Chassaing2005, Bergen2007, Schulz2005a (1/108 in PXE,	
c.3941G>A	p.R1314Q	M	m		E28	ICL9	+	1/46 in PXE relatives), Schulz2006, Hu2003c, Schulz2005b (1/108 in PXE, 1/46 in relatives)	170
c.3961G>A	p.G1321S	M	m	16248810	E28	ICL9	-	LeSaux 2001 (1/74 in US), Chassaing2005, Bergen2007, Hu2003c	171
				16248767				Chassaing 2004, Chassaing2005, RamsayM 2009 (2/48 PXE allles, 0.063 in	
c.4004T>C	p.L1335P	M	m		E28	ICL9		mixed South African pop.)	172
c.4004T>A	p.L1335Q	M	m	16248767	E28	ICL9		Pfendner 2007	173
								Pfendner 2007; Gheduzzi 2004 (3/98); LeSaux 2001 (0 in EU, 2.7 in US);	
								Miksch 2005 (11/170); Struk 2000, Uitto2001, Chassaing2005, Bergen2007,	
								LeSaux 2002 (18/34 in Afrikaner, 0 in other SA), Hu2003c, Ringpfeil2001,	
				16248756				Shi2007 (2/32), Faria 2013, RamsayM 2009 (39/95 PXE alleles, 0.411 in mixed	
								South African pop.), LaRUsso2010 (11.5% in mediterraneas, 9.1% of	
c.4015C>T	p.R1339C	M	m		E28	ICL9	+	scandinavians of mutated alleles)	174
c.4016G>A	p.R1339H	M	m	16248755	E28	ICL9	+	Pfendner 2007;	175
c.4016G>T	p.R1339L	M	m	16248755	E28	ICL9		Miksch 2005 (1/170)	176
c.4025T>C	p.I1342T	M	m	16248746	E28	ICL9		Pfendner 2007	177
c.4036C>T	p.P1346S	M	m	16248735	E28	ICL9	-	Chassaing 2005; Gheduzzi 2004 (1/98), Bergen2007	178
								LeSaux 2001 (0 in EU, 2.7% in US, probably also a splice site	
								mutation), Pfendner 2007, Chassaing2005, Bergen2007, Hu2003c,	
				16248730				may also result in aberrant splicing, a similar mutation in ABCC7 does,	
								although both correct+mutant splice variants are expressed due to the	
c.4041G>C	p.Q1347H	M	m, pot.splice		E28	ICL9 (Q-loop)		use of a cryptic splice site (LeSaux2001)	179
c.4060G>C	p.G1354R	M	m	16248633	E29	ICL9		Pulkkinen2001, Chassaing2005, Uitto2001, Bergen2007, Hu2003c	180
c.4069C>T	p.R1357W	M	m	16248624	E29	ICL9	+	Miksch 2005 (1/170), Noji2004	181
c.4081G>A	p.D1361N	M	m	16248612	E29	ICL9	+	LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007, Hu2003c	182
				16248589				LeSaux2001 (0.9% in EU, 0 in US), Chassaing2005, Bergen2007, LeSaux2002 (1/14), RamsayM 2009 (1/47 alleles in mixed South	
c.4104delC	p.D1368Efs	M	del, fs		E29	ICL9		African PXE pop.)	183
c.4182G>T	p.K1394N	M	m	16248511	E29	ICL9		Hu2003b (1/118), Chassaing2005, Bergen2007, Hu2004 (1/152)	184
								Hu2003b (4/118), Chassaing2005, Bergen2007, Hu2004 (2/152), Schulz2005a	
				16248511				(3/108 in PXE, 0/400 in controls), Schulz2006, Schulz2005b (3/108),	
c.4182delG	p.N1394fsX8	M	del, fs		E29	ICL9		Vanakker2008	185
c.4192C>T	p.R1398X	M	n	16248501	E29	ICL9	+	LeSaux 2001 (1/116 in EU), Pfendner 2007, Chassaing2005,	186
				16248495				Chassaing 2004, Chassaing2005, Gheduzzi2004 (4/98), Bergen2007, Faria	
c.4198G>A	p.E1400K	M	m		E29	ICL9	+	2013,	187
								Hendig 2005 (1/260) may also result in aberrant splicing since it is the first	
				16244629				position of exon 30, but Hending's paper does not mention it; Schulz2005a	
c.4209C>A	p.S1403R	M	m, pot.splice		E30	ICL9 (Signature)		(1/108 in PXE, 0/556 in controls), Schulz2006	188
c.4213G>A	p.G1405S	M	m	16244625	E30	ICL9 (Signature)	-	Vanakker2008 (1/76)	189
				16244618				Uitto2001, LeSaux2001, Bergen2000, Hu2003b (1/118), Chassaing2005,	
c.4220insAGAA		M	ins, fs		E30	ICL9		Bergen2007, Hu2004 (1/152), Plomp2004, Hu2003c, Ringpfeil2001	190
c.4253G>A	p.R1418Q	M	m	16244585	E30	ICL9	+	Meyer2005	191
c.4271T>C	p.I1424T	M	m	16244567	E30	ICL9		LeSaux 2001 (1/74 in US), Chassaing2005, Bergen2007, Hu2003c	192
c.4306_4312del	p.T1436fs	M	fs	16244532_16244526	E30	ICL9_		Plomp 2008 (o/140 control chromosomes)	193
c.4318delA	p.1440fsX1464	M	del, fs	16244520	E30	ICL9		Chassaing 2005; Gheduzzi 2004 (2/98), Bergen2007	194
				16244068				Hendig 2005 (2/260) alters the aa. sequence of the C-terminus and results in the	
c.4434delA	p.R1479fsX25	M	del, fs		E31	ICL9		loss of the regular termination codon; Schulz2006	195
c.4441G>A	p.G1481S	M	m	16244061	E31	ICL9	+	Pfendner 2007	196
c.4501G>A	p.G1501S	M	m	16244001	E31	ICL9	-	Pfendner 2007	197
c.1-2787del	p.M1_R929 ! uncorrect	M	del	16317291_16267141	E01-21			exon 21	198
	p.I1000_S1403del;W1404fsX146			16259790_16288485				LeSaux 2001 (4.3% in EU, 28.4% in US); Ringpfeil 2001; Miksch 2005 (19/170)indicated as in frame large del but out of frame deletion,	
c2996_4208del	3 p.1000Wfs...	M	del					Shi2007 (2/32), Nitschke 2012	199
	A995del405	M	del					Hu2003b (14/118), Hu2004 (17/152)	200
					E23-29			Katona2005, Bergen2007, Audo2007, Schulz2005a (5/108 in PXE, 3/46 in PXE relatives), Ringpfeil2006, Schulz2006, Hu2003c, Ringpfeil2001, SatoN 2009 (del_exon23), RamsayM 2009 (del 23-29: 3/95 PXE alleles, 0.043 in mixed South African pop.), LaRusso2010 (18/140)	201
c.2997_4208del		M	del	16259789_16248485				Ringpfeil2001b, Hu2003a	202

del ABCC6 ABCC1	p.M1-V1503del	M	del		E01-31	Miksch 2005 (1/170)large deletion	203
MYH11,+ 5 genes	p.M1-V1503del	M	del		E01-31	Miksch 2005 (1/170)large deletion	204
MYH11,+ 7 genes	p.M1-V1503del	M	del		E01-31	Miksch 2005 (1/170)large deletion	205
delABCC6						LeSaux2001, Bergen2000, Hu2003b, Bergen2007, Hu2004 (2/152), Hu2003c	206
c.(?_-37)_(*568_?)del		M	del		delABCC6	Costrop 2010	207
del ABCC6 ABCC1 MYH11		M	del			Hu2003b, Meloni2001, Hu2003c	208
exon24_25		M	del		E24-25	Katona2005	209
c.1-3189_36+1181del		M	del	1632048_16316075	5'UTR-I1	Chassaing2007	210
c.37-?_219+?del		M	del	16315688-?_16315506+?del	E2del	Costrop 2010	211
c.2667-42 2787+1623del		M	del	16267303_16265518	I20-I21	Chassaing2007	212
c.999-?_1176+?del		M	del	16296035-?_16295858+?del	E9del	Costrop 2010	213
c.1091_1338+3149del		M	del	16295943_16290255	E9-I10	Chassaing2007	214
c.1780-?_1867+?del		M	del	16281068-?_16280981+?del	E14del	Costrop 2010	215
ABCC6 del15 (1867+1530_1943+949del)	fs,p.624X	M	del		E15	LeSaux2001, Chassaing2005, Bergen2007, LeSaux2002 (2/14), Hu2003c	216
c.3307-904_3506+660del		M	del	c.3307-904_3506+660del	E24del	Costrop 2010	217
c.3307-1006_3882+1582del		M	del	16256043_16253102del	E24-E27del	Costrop 2010	218
c.4209-?_4403+?del		M	del	16244629-?_16244435+?del	E30del	Costrop 2010	219
c.4209-?_4512+?del		M	del	16244629-?_16243990+?del	E30-E31	Costrop 2010	220

A	B	C	D	E	F	G	H	I	
sequence variation at DNA level	sequence variation at protein level	Mutation/ Polym.	type of mut. (exonic intronic)	genomic pos. according to ENSG00000091262.10; 2013. July	genomic region affected	protein region affected	C>T at 5'CpG	references (allele frequency data and comments)	
c.1-219A>C		P	i	16317510	5'flanking			Pulkkinen2001 (freq:0.950/0.050), also present in Psi1 - gene conv?, Schulz2006 (1/122 in PXE, 14/236 in controls)	1
c.1-132C>T		P	i	16317423	5'flanking		+	Pulkkinen2001 (freq:0.953/0.047), Schulz2006 (freq:2/122 in PXE, 13/236 in controls)	2
c.1-127C>T		P	i	16317418	5'flanking		-	Pulkkinen2001 (freq:0.857/0.143), Schulz2006 (freq: 12/122 in PXE, 34/236 in controls)	3
c.117A>G	p.V39V	P	s	16315608	E2	TMH1		LeSaux2001, also present in Psi2	4
				16315543				LeSaux2001 (although called a "neutral variant" this nucleotide change is only present in 1/244 alleles and in none of the control chromosomes), LeSaux2002 (1/48 in PXE, no control patients, found in a patient homozygous for R1339C, but	
c.182G>A	p.G61D	P	m		E2	ICL1	-	why does this mean this is a polymorphism?!)	5
c.345+12T>C		P	i	16313667	I3			Miksch2005 (intronic duplication)	6
				16313653				Miksch2005, RamsayM 2009 (!? Found in 1/10 white and 3/10 black healthy south african person, do not mention if heteroz. or homoz., in mixed South	
c.345+26C>T		P	i		I3		+	African pop.)	7
c.474+13G>A		P	i	16313398	I4		+	Miksch2005(intronic duplication)	8
c.474+43C>T		P	i	16313368	I4		-	Miksch2005(intronic duplication)	9
c.475-76A>C		P	i	16308382	I4			Miksch2005(intronic duplication)	10
c.475-45C>T		P	i	16308351	I4		-	Miksch2005	11
c.475-22T>C		P	i	16308328	I4			Miksch2005(intronic duplication)	12
c.549G>A	p.L183L	P	s	16308232	E5	TMH5	-	LeSaux2000, Miksch2005	13
c.600+23C>T		P	i	16308158	I5		-	Miksch2005	14
				16306059				Miksch2005, Pulkkinen2001, LeSaux2002 (2/48), Miksch2005, RamsayM 2009 (!? Found in 6/24 south african PXE patient, do not mention if heteroz. or	
c.645G>A	p.T215T	P	s		E6	ICL3	+	homoz.)	15
c.662+12C>T		P	i	16306030	I6		+	Miksch2005, Pulkkinen2001	16
c.662+114T>C		P	i	16305928	I6			Pulkkinen2001 (also a mutation in Psi1 - gene conversion?)	17
c.662+298C>G		P	i	16305744	I6			Pulkkinen2001	18
c.662+305T>G		P	i	16305737	I6			Pulkkinen2001	19
c.662+403T>A		P	i	16305639	I6			Pulkkinen2001	20
c.794+36A>C		P	i	16302549	I7			Miksch2005(intronic duplication)	21
				16297410				LeSaux2002 (found by chance, 2/48 in PXE, no controls!), Miksch2005(reports	
c.855C>T	p.T285T	P	s		E8	ICL3	+	this mutation as found in pseudogene1)	22
								Miksch2005, RamsayM 2009 (!? Found in 1/10 black healthy south african	
c.993G>T	p.L331L	P	s	16297272	E8	ECL4		person, do not mention if heteroz. or homoz.)	23
								RamsayM 2009 (!? Found in 1/10 black healthy south african person, do not	
c.998+13C>T		P	i	16297254	I8		-	mention if heteroz. or homoz.)	24
				16295957				Miksch2005 (also found in pseodog.1), Cai2001 (in LD with 1132C>T, sometimes	
c.1077A>G	p.S359S	P	s		E9	TMH7		also 1141T>C)	25
				16295893				Cai2001 (in LD with 1077A>G; 1132C>T, possible gene conversion), Schulz2005b, Miksch2005(reports this mutation as found in ABCC6 and in	
c.1141T>C	p.L381L	P	s		E9	ICL4		pseudogene1)	26
c.1176+6C>T		P	i, pot. splice	16295852	I9		-	Miksch2005	27
				16291983				Miksch2005, HapMap, LeSaux2002 (20/48 in PXE, no controls), Morcher2003, Wang2001 (0.42 in Caucasians), Gheduzzi2004, Meloni2001, Schulz2005b, Miksch2005, RamsayM 2009 (!? Found in 2/10 black 1/10 white healthy south african person, and in 1/24 PXE	
c.1233T>C	p.N411N	P	s		E10	ICL4		patient do not mention if heteroz. or homoz.)	28
				16291971				Hu2003b (est. freq. 33%), Miksch2005, LeSaux2002 (20/45 in PXE, no controls), Morcher2003, Wang2001 (0.42 in Caucasians), Gheduzzi2004, Schulz2005b,	
c.1245G>A	p.V415V	P	s		E10	ICL4	-	Miksch2005, RamsayM 2009 (!? Found in 2/10 black healthy south african person, do not mention if heteroz. or homoz.)	29
c.1283A>G	p.N428S	P	m	16291933	E10	TMH8		Sato et al 2009 (found in healthy individuals (2/300 control allele, 2 heterozygotes) in a study collecting patients with AS but not with the full diagnosys of PXE)	30
c.1312G>A	p.V438M	P	m	16291904	E10	TMH8	+	Sato et al 2009 (found in healthy individuals (1/300 control allele, 1 heterozygote) in a study collecting patients with AS but not with the full diagnosys of PXE)	31
				16291871				Miksch2005, RamsayM 2009 (!? Found in 5/10 black healthy south african	
c.1338+7C>G		P	i		I10			person, do not mention if heteroz. or homoz.)	32
c.1338+20C>G		P	i	16291858	I10			Miksch2005	33
c.1338+62G>C		P	i	16291816	I10			Miksch2005	34
c.1338+1383T>C		P	i	16290495	I10			HapMap	35
c.1338+1501G>A		P	i	16290377	I10		-	HapMap	36
c.1338+1548C>T		P	i	16290330	I10		+	HapMap	37
c.1338+2152G>T		P	i	16289726	I10			HapMap	38
c.1338+2281A>C		P	i	16289597	I10			HapMap	39
c.1338+2346A>G		P	i	16289532	I10			HapMap	40
c.1339-2347C>G		P	i	16289126	I10			HapMap	41
c.1339-1977G>A		P	i	16288756	I10		-	HapMap	42
c.1344G>A	p.L448L	P	s	16286774	E11	ECL5	-	RamsayM 2009 (!? Found in 2/10 black healthy south african person, do not mention if heteroz. or homoz.)	43
c.1431+73G>C		P	i	16286614	I11			LeSaux2002 (2/48)	44
c.1432-215A>G		P	i	16284439	I11			HapMap	45
				16284272				HapMap, RamsayM 2009 (!? Found in 8/10 white healthy south african person, and in 7/24 PXE patient, do not mention if heteroz. or	
c.1432-48G>A		P	i		I11		-	homoz.)	46
c.1432-45C>A		P	i	16284269	I11			LeSaux2002 (1/48)	47
				16284269				RamsayM 2009 (!? Found in 2/10 white healthy south african person,	
c.1432-45C>T		P	i		I11		-	do not mention if heteroz. or homoz.)	48
c.1432-41A>G		P	i	16284265	I11			LeSaux2000, HapMap, Miksch2005, LeSaux2002 (5/48)	49
c.1432-41A>C		P	i	16284265	I11			RamsayM 2009 (!? Found in1/10 black 2/10 white healthy south african person, a	50
c.1432-22C>A		P	i	16284246	I11			LeSaux2002 (1/48)	51
c.1636-240G>A		P	i	16283071	I12		+	HapMap	52
c.1636-112C>T		P	i	16282943	I12		+	HapMap	53
c.1779+381C>T		P	i	16282307	I13		+	HapMap	54
c.1780-86G>T		P	i	16281154	I13			Chassaing2004, HapMap	55
				16281007				LeSaux2000, LeSaux2001 (200/244 PXE alleles, 163/200 control chromosomes), HapMap, Chassaing2004, Hu2003b (est.freq.52%), Miksch2005 (no cosegregation with disease), LeSaux2002 (16/48, no segregation with PXE), Wang2001 (0.17 in Chinese; 0.27 in Oji-Cree; 0.32 in South Asians; 0.41 in Africans; 0.45 in Caucasians; 0.56 in Inuit), Gheduzzi2004, Miksch2005,Schulz2006, Schulz2005b, SatoN2009 (studied amongst patients having AS but not full PXE, found in 16/108 AS alleles and 66/150 control alleles), RamsayM 2009 (!? Found in 2/10 black 3/10 white healthy south african person, and	
c.1841T>C	p.V614A	P	m		E14	ICL6		in 15/24 PXE patient, do not mention if heteroz. or homoz.)	56
c.1867+60A>G		P	i	16280921	I14			HapMap	57
c.1867+205C>G		P	i	16280776	I14			HapMap	58
c.1867+576G>A		P	i	16280405	I14		-	HapMap	59
c.1868-823C>T		P	i	16279714	I14		-	HapMap	60
c.1868-392C>T		P	i	16279283	I14		-	HapMap	61
c.1868-157C>G		P	i	16279048	I14			HapMap	62
c.1868_92delG		P	i	16278983	I14			Chassainq2004, Gheduzzi2004	63
c.1868-90G>T		P	i	16278981	I14			Chassaing2004, Gheduzzi2004	64
c.1868-57G>A		P	i	16278948	I14		+	Chassainq2004, Miksch2005, Gheduzzi2004	65
c.1890C>G	p.T630T	P	s	16287869	E15	ICL6		Chassaing2004, Hu2003b (est.freq.22%), Miksch2005, LeSaux2002 (11/48), Morcher2003, Wang2001 (0.6 in Caucasians), Gheduzzi2004, Meloni2001, Schulz2005b, RamsayM 2009 (!? Found in 3/10 black 1/10 white healthy south african person, and in 6/24 PXE patient, do not mention if heteroz. or homoz.)	66

								LeSaux2001 (17/24 PXE alleles, ND in control chromosomes), HapMap, Chassaing2004, Hu2003b (24%), Miksch2005 (in LD with other PXE mutations), LeSaux2002 (11/48, no segregation with PXE), Morcher2003, Wang2001 (0.4 in Caucasians), Gheduzzi2004, Struk2000, Hu2003a (34/32 in PXE, 80/204 in controls), Schulz2005b, SatoN2009 (studied amongst patients having AS but not full PXE, found in 33/108 AS alleles and 65/150 control alleles), RamsayM 2009 (!? Found in 3/10 black 1/10 white healthy south african person, do	
c.1896C>A	p.H632Q	P	m	16287863	E15	ICL6		not mention if heteroz. or homoz.)	67
c.1943+121T>A		P	i	16278695	I15			HapMap	68
c.1943+766G>A		P	i	16278050	I15		-	HapMap	69
c.1944-898C>T		P	i	16277685	I15		-	HapMap	70
c.1944-177G>A		P	i	16276964	I15		-	HapMap	71
				16276345				Hendig 2005 (3/260 PXEp. - 2/200 control), Miksch2005 (no cosegregation with disease), Schulz2005a (7/674), Ramsay 2009 (uncorrect protein alteration	
c.2171G>A	p.R724K	P	m		E17	ICL6	-	reported)	72
				16276341				Hendig 2005 (3/260 PXEp. - 2/200 control), Miksch2005, Schulz2005a (7/674), RamsayM 2009 (Found in 2/10 blacksouth african pop. , do not mention if	
c.2175A>T	p.V725V	P	s		E17	ICL6		heteroz. or homoz.)	73
				16276292				Hendig 2005 (3/260 PXEp. - 2/200 control), Miksch2005 (no cosegregation with disease), Schulz2005a (7/674) RamsayM 2009 (Found in 1/10 black south african pop. , do not mention if heteroz. or	
c.2224A>G	p.I742V	P	m		E17	ICL6		homoz.)	74
c.2247+302C>T		P	i	16275967	I17		+	HapMap	75
c.2247+857C>T		P	i	16275412	I17		-	HapMap	76
c.2248-1384G>C		P	i	16274206	I17			HapMap	77
c.2248-552G>A		P	i	16273374	I17		+	HapMap	78
				16272670				HapMap, RamsayM 2009 (Found in 2/10 black , 1/24 PXE patients in south	
c.2400A>G	p.G800G	P	s		E18	ICL6		african pop.do not mention if heteroz. or homoz.)	79
c.2415+147C>T		P	i	16272508	I18		+	HapMap	80
				16271409				Hendig 2005 (allele frequency data was not clear); LeSaux 2000; Hu 2003, HapMap, Miksch2005, Ringpfeil2001b, LeSaux2002 (6/48), Morcher2003, Wang2001 (0.08 in Caucasians), Gheduzzi2004, Schulz2006, Schulz2005b, RamsayM 2009 (Found in 3/24 PXE patient in south african pop., do not	
c.2490C>T	p.A830A	P	s		E19	ICL6	-	mention if heteroz. or homoz.)	81
				16271357				HapMap, RamsayM 2009 (Found in 3/10 black healthy , 1/24 PXE patient in	
c.2542A>G	p.M848V	P	m		E19	ICL6		south african pop, do not mention if heteroz. or homoz.)	82
c.2590+405G>A		P	i	16270904	I19		-	HapMap	83
c.2590+459G>A		P	i	16270850	I19		-	HapMap	84
c.2591-331A>G		P	i	16270174	I19			HapMap	85
c.2591-203T>C		P	i	16270046	I19			HapMap	86
c.2591-119C>T		P	i	16269962	I19		-	HapMap	87
c.2631C>A	p.T877T	P	s	16269803	E20	ICL6		Morcher2003(present in several non-PXE people)	88
c.2666+133_134insC		P	i	16269634_16269635	I20			Hendiq2005 (4/260)	89
c.2666+198T>C		P	i	16269570	I20			HapMap	90
c.2666+499C>T		P	i	16269269	I20		+	HapMap	91
c.266+900C>T		P	i	16268868	I20		+	HapMap	92
c.2787+30G>A		P	i	16267111	I21		+	LeSaux2002 (2/48)	93
c.2787+62T>C		P	i	16267079	I21			HapMap	94
c.2787+1427C>G		P	i	16265714	I21			HapMap	95
c.2787+1580G>T		P	i	16265561	I21			HapMap	96
c.2788-706T>C		P	i	16264416	I21			HapMap	97
c.2788-388A>C		P	i	16264098	I21			HapMap	98
c.2788-127A>G		P	i	16263837	I21			HapMap	99
c.2820T>G	p.R940R	P	s	16263678	E22	TMH12		Miksch2005	100
				16263663				Hendig 2005 (7/240); LeSaux 2002, HapMap, Morcher2003, RamsayM 2009 (Found in 2/10 black healthy, 1/10 white healthy, 1/24 PXE patient in south african pop, do not mention if heteroz. or	
c.2835C>T	p.P945P	P	s		E22	TMH12	-	homoz.)Miksch2005	101
				16263662				Hendig 2005(3/260 PXEp. - 7/200 control); Morcher 2003 (M), Miksch2005 (P, no cosegregation with disease), Shi2007, Hendig (P, because present in normal pop) vs Morcher (M), RamsayM 2009 (Found in 1/24 PXE patient in south african pop, do not mention if	
c.2836C>A	p.L946I	P	m		E22	TMH12		heteroz. or homoz.)	102
c.2904G>A	p.L968L	P	s	16263594	E22	ECL7	-	Hu2003b (est.freq.20%), Miksch2005	103
c.2995+142C>T		P	i	16263361	I22		+	HapMap	104
c.2995+931C>T		P	i	16262572	I22		-	HapMap	105
c.2995+1329G>A		P	i	16262174	I22		-	HapMap	106
c.2996-1461G>A		P	i	16261251	I22		-	HapMap	107
c.2996-1380C>G		P	i	16261170	I22			HapMap	108
c.2996-1353G>A		P	i	16261143	I22		-	HapMap	109
c.2996-1213G>A		P	i	16261003	I22		-	HapMap	110
c.2996-1028G>T		P	i	16260818	I22			HapMap	111
c.2996-653T>C		P	i	16260443	I22			HapMap	112
c.2996-637G>A		P	i	16260427	I22		+	HapMap	113
c.2996-552G>A		P	i	16260342	I22		+	HapMap	114
c.3190C>T	p.R1064W	P	m	16259596	E23	TMH14	+	Miksch2005 (does not cosegregate with disease), Morcher2003	115
c.3306+531C>T		P	i	16258949	I23		+	HapMap	116
c.3306+650A>G		P	i	16258830	I23			HapMap	117
c.3306+855G>T		P	i	16258625	I23			HapMap	118
c.3307-1124C>T		P	i	16258173	I23		+	HapMap	119
c.3307-897A/C/G/T		P	i	16257946	I23			HapMap	120
c.3307-361G>T		P	i	16257410	I23			HapMap	121
c.3508+83C>A		P	-	16256767	I24			Meloni2001, HapMap, Bowen2007	122
c.3508+172C>T		P	-	16256678	I24		-	HapMap	123
				16255437				Miksch2005, HapMap, LeSaux2002 (1/48), RamsayM 2009 (Found in 2/10 black healthy, 3/24 PXE patient in south african pop, do not	
c.3507-16T>C		P	-		I24			mention if heteroz. or homoz.)	124
c.3633+55T>C		P	i	16255240	I25			LeSaux2002 (8/48), HapMap	125
c.3633+90G>A		P	i	16255205	I25		-	LeSaux2000, LeSaux2002 (21/48)	126
c.3633+529A>G		P	i	16254766	I25			HapMap	127
c.3634-506G>A		P	i	16253946	I25		-	HapMap	128
c.3634-394C>T		P	i	16253834	I25		-	HapMap	129
c.3634-130G>A		P	i	16253570	I25		-	HapMap	130
c.3735+643A>G		P	i	16252696	I26			HapMap	131
c.3736-716G>T		P	i	16252382	I26			HapMap	132
c.3736-502C>T		P	i	16252168	I26		+	HapMap	133
c.3736-334A>C		P	i	16252000	I26			HapMap	134
c.3736-233A>C		P	i	16251899	I26			HapMap	135
				16251599				LeSaux2001 (23/244 of PXE chromosomes, 31/200 control chromosomes), HapMap, Miksch2005 (no segregation with disease+in LD with other PXE mut), LeSaux2002 (4/48 - no segregation with disease), Morcher2003, Wang2001 (0.04 in Africans; 0.06 in Chinese; 0.14 in Oji-Cree; 0.16 in South Asians; 0.17 in Inuit; 0.30 in Caucasians), Ringpfeil2000 (listed as mut.; 3/16), Gheduzzi2004, Hu2003a (G:0.3; A:0.7), Schulz2006, Germain2000 (24/124), Meyer2005, Schulz2005b (15/108 in PXE, 14/46 in relatives), SatoN2009 (found in healthy (48/300 control alleles) and AS (34/108 AS alleles) individuals in a study collecting patients with AS but not with the full diagnosys of PXE), QiaoliLi2011 (in association with the	
c.3803G>A	p.R1268Q	P	m		E27	ICL9	+	development of AS)	136
c.3882+531C>T		P	i	16250989	I27		-	HapMap	137
c.3882+922G>C		P	i	16250598	I27			HapMap	138
c.3883-46A>G		P	i	16248934	I27			LeSaux2000	139
c.3883-24G>A		P	i	16248912	I27		-	Miksch2005	140
c.3978C>T	p.D1326D	P	s	16248793	E28	ICL9	+	Wang2001 (0.03 in Caucasians)	141
c.4041+49C>T		P	i	16248681	I28		+	Miksch2005	142

				16248681				LeSaux2000, HapMap, Miksch2005, LeSaux2002 (18/48), Schulz2005a (?), Bowen2007, Shi2007 (3/32), in LD with 3940C>T?	
c.4042-30C>T		P	i		I28		+	(Shi2007)	143
c.4208+9G>A		P	i	16248476	I29		+	Miksch2005, Schulz2005a (5/266 in AAA)	144
c.4208+1014T>A		P	i	16247471	I29			HapMap	145
c.4209-1100C>T		P	i	16245729	I29		+	HapMap	146
c.4254G>A	p.R1418R	P	s	16244584	E30	ICL9	-	Schulz2005a (6/266 in AAA; 2/244 in controls)	147
c.4305C>T	p.G1435G	P	s	16244533	E30	ICL9	-	Miksch2005	148
c.4403+11C>G		P	i	16244424	I30			Hendig2005 (2/260), Schulz2005a (2/108 in PXE, 0 in controls)	149
c.4404-81C>T		P	i	16244179	I30		+	Hendig (1/260)	150
c.4404-76A>G		P	i	16244174	I30			Chassaing2004, HapMap, Hendig2005, Gheduzzi2004, Schulz2006	151
c.4404-31G>A		P	i	16244129	I30		-	Hendig2005, HapMap, Miksch2005, Gheduzzi2004, Schulz2006	152
c.4512+17G>A		P	i	16243973	3'UTR		+	LeSaux2000, Miksch2005, LeSaux2002 (2/48)	153
c.4512+38G>A		P	i	16243952	3'UTR		+	Miksch2005	154

Supp.Tabble 4

A	B	C	D	E	F	G	H	I	
sequence variation at DNA level	**sequence variation at protein level**	**Mutation/ Polym.**	**type of mut. (exonic intronic)**	**genomic pos. (according to ENSG00000091262.10; 2013. July)**	**genomic region affected**	**protein region affected**	**C>T at 5'CpG**	**references (allele frequency data and comments)**	
c.105delA	p.S37fsX80	M	del, fs	16315620	E2	TMH1-TMH2		Miksch 2005 (1/170)	1
c.175_179del	p.G58fs	M	del, fs	16315550_16315546	E2	ICL1		Vanakker (3/76)	2
c.179_187del	p.R60_Y62del	M	del	16315546_16315538	E2	ICL1		Pulkkinen 2001;Miksch 2005 (1/170) in frame del; Chassaing2005, Bergen2007, Hu2003c	3
c.179_195del		M	del, fs	16315546_16315530	E2	ICL1		LeSaux2001 (0.9% in EU, 0 in US), Chassaing2005, Bergen2007, Hu2003c	4
c.220_222del	p.V74del	M	del	16313804_16313802	E3	TMH2		Chassaing 2004 also a splice mutation since this is the first codon of exon 3, Chassaing2005, Bergen2007	5
c.450_451insC	p.A151Rfs	M	ins, fs	16313435_16313434	E4	ECL3_		Nitschke 2012	6
c.708_709dupCT	p.W237Sfs*21	M	dup	16302671_16302670	ICL3	ICL3		Tan2012 (case report, homozygous mutation in this patient, born from consanguineous parents, no controls)	7
c.865_889del		M	del, fs	16297400_16297376	E8	ICL3		LeSaux2002 (found in a heterozygous state in an apparently healty (control) person)	8
c.938_939insT		M	ins, fs	16297327_16297326	E8	TMH6		LeSaux2001 (1/202), Chassaing2005, Bergen2007, LeSaux2002 (1/34 in Afrikaner, 1/14 in other SA), Hu2003c	9
c.960delC	p.I320fs	M	del, fs	16297305	E8	TMH6		Chassaing 2005; Meloni 2001, Bergen2007, Hu2003c	10
c.1088_1120del	p.363_374QTLF EQQNMYRdel	M	del	16295946_16295914	E9	TMH7-ICL4		Chassaing 2004 in frame deletion, Chassaing2005, Bergen2007, MartinL 2008	11
c.1574_1575insG	p.L525fsX73	M	ins, fs	16284082_16284081	E12	ICL5		Schulz2006	12
c.1674delC	p.A558fs	M	del, fs	16282793	E13	ECL6_		Vanakker2008 (1/76)	13
c.1857_1858insC		M	fs	16280991_16280990	E14	ICL6_		Pfendner 2007	14
c.1944_1965del	p648fsX688	M	del, fs	16276787_16276766	E16	ICL6 (WalkerA)		LeSaux2001, Hu2003b (2/118), Bergen2000, Hu2004 (2/152), Hu2003a, Hu2003c, Ringpfeil2001	15
c.1967_1989del		M	fs	16276764_16276742	E16	ICL6_		Bergen 2000	16
c.1995delG	p.A667fsX20	M	del, fs	16276736	E16	ICL6 (WalkerA)		LeSaux2001 (0.9% in EU, 0 in US), Hendig2005 (3/152), Chassaing2005, Bergen2007, Schulz2005a (3/108 in PXE patients), Schulz2006, Hu2003c, Schulz2005b	17
c.2237_2238ins10		M	fs	16276279_16276278	E17	ICL6_		Pfendner 2007	18
c.2322delC	p.774Yfs	M	del, fs	16272748	E18	ICL6_		LeSaux2001 (1/74 in US), Chassaing2005, Bergen2007, Hu2003c	19
c.2542delG	p.2848fs	M	del, fs	16271357	E19	ICL6		Uitto2001, LeSaux2001, Chassaing2005, Bergen2007, Noji2004, Hu2003c, Ringpfeil2001, according to reference genome there is an A at this position, but this is also a HapMap polymorphism, with A being the minor allele only present in yoruba and japanese, SatoN2009 (associates with angioid streaks, in a study collecting patients with AS but not with the full diagnosys of PXE, 27/108 AS allele and 1/300 control allele), La Russo2010(onlz present in asians, japanese)	20
c.2820insC		M	fs	14263678	E22	TMH12_		Pfendner 2007	21
c.2835 _2850del16	p.P946fsX17	M	del, fs	16263663_16263648	E22	TMH12		Hendig 2005 (1/108) out of frame deletion that results in 17 new codons and premature STOP, Schulz2006	22
c.3105_3107delCTT	p.F1036del	M	del	16259681_16259679	E23	ICL7		Nitschke 2012	23
c.3106_3108delTTT	p.F1036del	M	del	16259680_16259678	E23	ICL7		Miksch 2005 (1/170)in frame del	24
c.3141_3143delTCT	p.F1048del	M	del	16259645_16259643	E23	ICL7		Miksch 2005 (1/170)	25
c.3364delT	p.S1122Lfs	M	fs	16256992	E24	ICL8		Vanakker2008 (1/76)	26
c.3544dupC	p.L1182PfsX127 8	M	dup, fs	16255384	E25	TMH16_		Chassaing 2005, Bergen2007, Gheduzzi2004 (1/98)	27
c.3769_3770insC	p.L1259fs	M	ins, fs	16251633_16251632	E27	ICL9		Miksch 2005 (2/170)	28
c.3775delT	p.W1259Gfs	M	del, fs	16251627	E27	ICL9		LeSaux2000 (causes X at 3815-3817), Uitto2001, LeSaux2001 (1.7% in EU, 0 in US), Bergen2000, Hu2003b (8/118), Miksch2005 (2/170), Chassaing2005, Bergen2007, LeSaux2002 (1/14), Hu2004 (11/152), Hu2003a, Schulz2006, Hu2003c, Ringpfeil2001, Schulz2005b (1/108), RamsayM 2009 (1/47 alleles in mixed South African PXE pop.), Plomp 2009	29
c.3798delT		M	fs	16251604	E27	ICL9		Bergen2007	30
c.3821_3868del48	p.1274Ydel16	M	del	16251581_15251534	E27	ICL9		Hu2004 (1/152)	31
c.3912delG	p.S1305fsX1358	M	del, fs	16248859	E28	ICL9		Miksch 2005 (1/170)	32
c.4104delC	p.D1368Efs	M	del, fs	16248589	E29	ICL9		LeSaux2001 (0.9% in EU, 0 in US), Chassaing2005, Bergen2007, LeSaux2002 (1/14), RamsayM 2009 (1/47 alleles in mixed South African PXE pop.)	33
c.4182delG	p.N1394fsX8	M	del, fs	16248511	E29	ICL9		Hu2003b (4/118), Chassaing2005, Bergen2007, Hu2004 (2/152), Schulz2005a (3/108 in PXE, 0/400 in controls), Schulz2006, Schulz2005b (3/108), Vanakker2008	34
c.4220insAGAA		M	ins, fs	16244618	E30	ICL9		Bergen2007, Hu2004 (1/152), Plomp2004, Hu2003c, Ringpfeil2001	35
c.4306_4312del	p.T1436fs	M	fs	16244532_16244526	E30	ICL9_		Plomp 2008 (o/140 control chromosomes)	36
c.4318delA	p.1440fsX1464	M	del, fs	16244520	E30	ICL9		Chassaing 2005; Gheduzzi 2004 (2/98), Bergen2007	37
c.4434delA	p.R1479fsX25	M	del, fs	16244068	E31	ICL9		Hendig 2005 (2/260) alters the aa. sequence of the C-terminus and results in the loss of the regular termination codon; Schulz2006	38

small deletions
insertions
duplications

Supp.Table 5

A	B	C	D	F	G	H	
sequence variation at DNA level	sequence variation at protein level	Mutation/ Polym.	type of mut. (exonic intronic)	genomic region affected	protein region affected	C>T at 5'CpG	
c.113G>C	p.W38S	M	m	E2	TMH1		1
c.373G>A	p.E125K	M	m	E4	ICL2		2
c.386G>A	p.G129E	M	m	E4	ICL2		3
c.496C>T	p.R166C	M	m	E5	ECL3	+	4
c.676G>A	p.G226R	M	m	E7	ICL3	-	5
c.754C>T	p.L252F	M	m	E7	ICL3	-	6
c.951C>G	p.S317R	M	m	E8	TMH6		7
c.951C>A	p.S317R	M	m	E8	TMH6		8
c.1064T>G	p.L355R	M	m	E9	TMH7		9
c.1091C>G	p.T364R	M	m	E9	TMH7		10
c.1091C>T	p.T364M	M	m	E9	TMH7	+	11
c.1108A>G	p.N370D	M	m	E9	TMH7		12
c.1144C>T	p.R382W	M	m	E9	ICL4	+	13
c.1171A>G	p.R391G	M	m	E9	ICL4		14
c.1176G>C	p.K392N	M	m	E9	ICL4		15
c.1192A>G	p.S398G	M	m	E10	ICL4		16
c.1194C>G	p.S398R	M	m	E10	ICL4		17
c.1233T>G	p.N411K	M	m	E10	ICL4		18
c.1244T>C	p.V415A	M	m	E10	ICL4		19
c.1318T>G	p.C440G	M	m	E10	TMH8		20
c.1363G>C	p.A455P	M	m	E11	TMH9		21
c.1388T>A	p.L463H	M	m	E11	TMH9		22
c.1396A>T	p.N466Y	M	m	E11	TMH9		23
c.1460G>A	p.R487Q	M	m	E12	ICL5	+	24
c.1484T>A	p.L495H	M	m	E12	ICL5		25
c.1491C>A	p.N497K	M	m	E12	ICL5		26
c.1498A>C	p.T500P	M	m	E12	ICL5		27
c.1505A>T	p.K502M	M	m	E12	ICL5		28
c.1553G>A	p.R518Q	M	m	E12	ICL5	+	29
c.1563G>C	p.E521D	M	m	E12	ICL5		30
c.1603T>C	p.S535P	M	m	E12	TMH10		31
c.1652T>C	p.F551S	M	m	E13	TMH10		32
c.1685T>C	p.M562T	M	m	E13	ECL6		33
c.1703T>C	p.F568S	M	m	E13	ICL6		34
c.1769C>T	p.S590F	M	m	E13	TMH11	-	35
c.1781C>T	p.A594V	M	m	E14	TMH11	-	36
c.1798C>T	p.R600C	M	m	E14	ICL6	+	37
c.1987G>T	p.G663C	M	m	E16	ICL6 (WalkerA)		38
c.2018T>C	p.L673P	M	m	E16	ICL6		39
c.2030T>C	p.L677P	M	m	E16	ICL6		40
c.2093A>C	p.Q698P	M	m	E17	ICL6 (Q-loop)		41
c.2097G>T	p.E699D	M	m	E17	ICL6		42
c.2177T>C	p.L726P	M	m	E17	ICL6		43
c.2252T>A	p.M751K	M	m	E18	ICL6		44
c.2263G>A	p.G755R	M	m	E18	ICL6 (Signature)	+	45
c.2278C>T	p.R760W	M	m	E18	ICL6 (Signature)	+	46
c.2279G>A	p.R760Q	M	m	E18	ICL6 (Signature)	+	47
c.2293C>T	p.R765W	M	m	E18	ICL6 (Signature)	+	48
c.2294G>A	p.R765Q	M	m	E18	ICL6 (Signature)	+	49
c.2297C>A	p.A766D	M	m	E18	ICL6 (Signature)		50
c.2329G>A	p.D777N	M	m	E18	ICL6 (WalkerB	-	51
c.2342C>T	p.A781V	M	m	E18	ICL6 (WalkerB	+	52
c.2379C>G	p.N793L	M	m	E18	ICL6		53
c.2419C>T	p.R807W	M	m	E19	ICL6	+	54
c.2420G>A	p.R807Q	M	m	E19	ICL6	+	55
c.2428G>A	p.V810M	M	m	E19	ICL6	+	56

c.2432C>T	p.T811M	M	m	E19	ICL6	+	57
c.2458G>C	p.A820P	M	m	E19	ICL6		58
c.2477T>C	p.L826P	M	m	E19	ICL6		59
c2552T>C	p.L851P	M	m	E19	ICL6		60
c.2643G>T	p.R881S	M	m	E20	ICL6		61
c.2831C>T	p.T944I	M	m	E22	TMH12	-	62
c.2848G>A	p.A950T	M	m	E22	TMH12	+	63
c.2891G>C	p.R964P	M	m	E22	ECL7		64
c.2943G>T	p.Q981H	M	m	E22	ECL7		65
c.2974G>C	p.G992R	M	m	E22	ECL7		66
c.3074T>C	p.L1025P	M	m	E23	ICL7		67
c.3145T>G	p.S1049A	M	m	E23	ICL7		68
c.3168C>A	p.D1056E	M	m	E23	ICL7		69
c.3188T>G	p.L1063R	M	m	E23	TMH14		70
c.3340C>T	p.R1114C	M	m	E24	ICL8	+	71
c.3341G>C	p.R1114P	M	m	E24	ICL8		72
c.3341G>A	p.R1114H	M	m	E24	ICL8	+	73
c.3362C>T	p.S1121L	M	m	E24	ICL8	+	74
c.3362C>G	p.S1121W	M	m	E24	ICL8		75
c.3389C>T	p.T1130M	M	m	E24	ICL8	+	76
c.3397G>T	p.G1133C	M	m	E24	ICL8		77
c.3398G>C	p.G1133A	M	m	E24	ICL8		78
c.3412C>T	p.R1138W	M	m	E24	ICL8	+	79
c.3413G>A	p.R1138Q	M	m	E24	ICL8	+	80
c.3413G>C	p.R1138P	M	m	E24	ICL8		81
c.3415G>A	p.A1139T	M	m	E24	ICL8	-	82
c.3491G>A	p.R1164Q	M	m	E24	ICL8	+	83
c.3608G>A	p.G1203D	M	m	E25	TMH17	-	84
c.3661C>T	p.R1221C	M	m	E26	ICL9	+	85
c.3662G>A	p.R1221H	M	m	E26	ICL9	+	86
c.3676C>A	p.L1226I	M	m	E26	ICL9		87
c3703C>T	p.R1235W	M	m	E26	ICL9	+	88
c3712G>C	p.D1238H	M	m	E26	ICL9		89
c.3715T>C	p.Y1239H	M	m	E26	ICL9		90
c.3735G>T	p.E1245D	M	m, pot.splice	E26	ICL9		91
c.3735G>A	p.E1245E	M	s, pot. splice	E26	ICL9	-	92
c.3787G>A	p.G1263R	M	m	E27	ICL9	+	93
c.3818G>A	p.R1273K	M	m	E27	ICL9	-	94
c.3877G>A	p.E1293K	M	m	E27	ICL9	-	95
c.3892G>T	p.V1298F	M	m	E28	ICL9 (WalkerA)		96
c3895G>A	p.G1299S	M	m	E28	ICL9 (WalkerA)	-	97
c.3902C>T	p.T1301I	M	m	E28	ICL9 (WalkerA)	-	98
c.3904G>A	p.G1302R	M	m	E28	ICL9 (WalkerA)	+	99
c.3907G>C	p.A1303P	M	m	E28	ICL9 (WalkerA)		100
c.3919T>C	p.S1307P	M	m	E28	ICL9 (WalkerA)		101
c.3932G>A	p.G1311E	M	m	E28	ICL9	-	102
c.3940C>T	p.R1314W	M	m	E28	ICL9	+	103
c.3941G>A	p.R1314Q	M	m	E28	ICL9	+	104
c.3961G>A	p.G1321S	M	m	E28	ICL9	-	105
c.4004T>C	p.L1335P	M	m	E28	ICL9		106
c.4004T>A	p.L1335Q	M	m	E28	ICL9		107
c.4015C>T	p.R1339C	M	m	E28	ICL9	+	108
c.4016G>A	p.R1339H	M	m	E28	ICL9	+	109
c.4016G>T	p.R1339L	M	m	E28	ICL9		110
c.4025T>C	p.I1342T	M	m	E28	ICL9		111
c.4036C>T	p.P1346S	M	m	E28	ICL9	-	112
c.4041G>C	p.Q1347H	M	m, pot.splice	E28	ICL9 (Q-loop)		113
c.4060G>C	p.G1354R	M	m	E29	ICL9		114
c.4069C>T	p.R1357W	M	m	E29	ICL9	+	115
c.4081G>A	p.D1361N	M	m	E29	ICL9	+	116
c.4182G>T	p.K1394N	M	m	E29	ICL9		117

c.4198G>A	p.E1400K	M	m	E29	ICL9	+	118
c.4209C>A	p.S1403R	M	m, pot.splice	E30	ICL9 (Signature)		119
c.4213G>A	p.G1405S	M	m	E30	ICL9 (Signature)	-	120
c.4253G>A	p.R1418Q	M	m	E30	ICL9	+	121
c.4271T>C	p.I1424T	M	m	E30	ICL9		122
c.4441G>A	p.G1481S	M	m	E31	ICL9	+	123
c.4501G>A	p.G1501S	M	m	E31	ICL9	-	124
c.373G>T	p.E125X	M	n	E4	ICL2_		125
c.595C>T	p.Q199X	M	n	E5	ICL3	-	126
c.681C>G	p.Y227X	M	n	E7	ICL3		127
c.724G>T	p.E242X	M	n	E7	ICL3		128
c.1087C>T	p.Q363X	M	n	E9	TMH7	-	129
c.1132C>T	p.Q378X	M	n	E9	ICL4	-	130
c.1552C>T	p.R518X	M	n	E12	ICL5	+	131
c.2162G>A	p.W721X	M	n	E17	ICL6	-	132
c.2245C>T	p.Q749X	M	n	E17	ICL6	-	133
c.2304C>A	p.Y768X	M	n	E18	ICL6		134
c.2511C>A	p.Y837X	M	n	E19	ICL6		135
c.2524C>T	p.Q842X	M	n	E19	ICL6	-	136
c.2814C>G	p.Y938X	M	n	E22	ICL6		137
c.3088C>T	p.R1030X	M	n	E23	ICL7	+	138
c.3207C>A	p.Y1069X	M	n	E23	TMH14		139
c.3421C>T	p.R1141X	M	n	E24	ICL8	+	140
c.3427C>T	p.Q1143X	M	n	E24	ICL8	-	141
c.3490C>T	p.R1164X	M	n	E24	ICL8	+	142
c.3668G>A	p.W1223X	M	n	E26	ICL9	-	143
c.3709C>T	p.Q1237X	M	n	E26	ICL9	-	144
c.3722G>A	p.W1241X	M	n	E26	ICL9	-	145
c.3823C>T	p.R1275X	M	n	E27	ICL9	+	146
c.4192C>T	p.R1398X	M	n	E29	ICL9	+	147

Supp.Table 6

A	B	C	D	E	F	G	H	I	
sequence variation at DNA level	sequence variation at protein level	Mutation/ Polym.	type of mut. (exonic intronic)	genomic pos. (according to ENSG00000091262.10; 2013. July)	genomic region affected	protein region affected	C>T at 5'CpG	references (allele frequency data and comments)	
c.117A>G	p.V39V	P	s	16315608	E2	TMH1		LeSaux2001, also present in Psi2	1
				16315543				LeSaux2001 (although called a "neutral variant" this nucleotide change is only present in 1/244 alleles and in none of the control chromosomes), LeSaux2002 (1/48 in PXE, no control patients, found in a patient homozygous for R1339C, but	
c.182G>A	p.G61D	P	m		E2	ICL1	-	why does this mean this is a polymorphism?!)	2
c.549G>A	p.L183L	P	s	16308232	E5	TMH5	-	LeSaux2000, Miksch2005	3
				16306059				Miksch2005, Pulkkinen2001, LeSaux2002 (2/48), Miksch2005, RamsayM 2009 (!? Found in 8/24 south african PXE patient, do not mention if heteroz. or	
c.645G>A	p.T215T	P	s		E6	ICL3	+	homoz.)	4
				16297410				LeSaux2002 (found by chance, 2/48 in PXE, no controls!), Miksch2005(reports	
c.855C>T	p.T285T	P	s		E8	ICL3	+	this mutation as found in pseudogene1)	5
c.993G>T	p.L331L	P	s	16297272	E8	ECL4		Miksch2005, RamsayM 2009 (!? Found in 1/10 black healthy south african person, do not mention if heteroz. or homoz.)	6
				16295957				Miksch2005 (also found in pseodog.1), Cai2001 (in LD with 1132C>T, sometimes	
c.1077A>G	p.S359S	P	s		E9	TMH7		also 1141T>C)	7
				16295893				Cai2001 (in LD with 1077A>G; 1132C>T, possible gene conversion), Schulz2005b, Miksch2005(reports this mutation as found in ABCC6 and in	
c.1141T>C	p.L381L	P	s		E9	ICL4		pseudogene1)	8
				16291983				Miksch2005, HapMap, LeSaux2002 (20/48 in PXE, no controls), Morcher2003, Wang2001 (0.42 in Caucasians), Gheduzzi2004, Meloni2001, Schulz2005b, Miksch2005, RamsayM 2009 (!? Found in 2/10 black 1/10 white healthy south african person, and in 1/24 PXE	
c.1233T>C	p.N411N	P	s		E10	ICL4		patient do not mention if heteroz. or homoz.)	9
				16291971				Hu2003b (est. freq. 33%), Miksch2005, LeSaux2002 (20/45 in PXE, no controls), Morcher2003, Wang2001 (0.42 in Caucasians), Gheduzzi2004, Schulz2005b,	
c.1245G>A	p.V415V	P	s		E10	ICL4	-	Miksch2005, RamsayM 2009 (!? Found in 2/10 black healthy south african person, do not mention if heteroz. or homoz.)	10
c.1283A>G	p.N428S	P	m	16291933	E10	TMH8		Sato et al 2009 (found in healthy individuals (2/300 control allele, 2 heterozygotes) in a study collecting patients with AS but not with the full diagnosys of PXE)	11
c.1312G>A	p.V438M	P	m	16291904	E10	TMH8	+	Sato et al 2009 (found in healthy individuals (1/300 control allele, 1 heterozygote) in a study collecting patients with AS but not with the full diagnosys of PXE)	12
c.1344G>A	p.L448L	P	s	16286774	E11	ECL5	-	RamsayM 2009 (!? Found in 2/10 black healthy south african person, do not mention if heteroz. or homoz.)	13
				16281007				LeSaux2000, LeSaux2001 (200/244 PXE alleles, 163/200 control chromosomes), HapMap, Chassaing2004, Hu2003b (est.freq.52%), Miksch2005 (no cosegregation with disease), LeSaux2002 (16/48, no segregation with PXE), Wang2001 (0.17 in Chinese; 0.27 in Oji-Cree; 0.32 in South Asians; 0.41 in Africans; 0.45 in Caucasians; 0.56 in Inuit), Gheduzzi2004, Miksch2005,Schulz2006, Schulz2005b, SatoN2009 (studied amongst patients having AS but not full PXE, found in 16/108 AS alleles and 66/150 control alleles), RamsayM 2009 (!? Found in 2/10 black 3/10 white healthy south african person, and	
c.1841T>C	p.V614A	P	m		E14	ICL6		in 15/24 PXE patient, do not mention if heteroz. or homoz.)	14
c.1890C>G	p.T630T	P	s	16287869	E15	ICL6		Chassaing2004, Hu2003b (est.freq.22%), Miksch2005, LeSaux2002 (11/48), Morcher2003, Wang2001 (0.6 in Caucasians), Gheduzzi2004, Meloni2001, Schulz2005b, RamsayM 2009 (!? Found in 3/10 black 1/10 white healthy south african person, and in 6/24 PXE patient, do not mention if heteroz. or homoz.)	15
c.1896C>A	p.H632Q	P	m	16287863	E15	ICL6		LeSaux2001 (17/24 PXE alleles, ND in control chromosomes), HapMap, Chassaing2004, Hu2003b (24%), Miksch2005 (in LD with other PXE mutations), LeSaux2002 (11/48, no segregation with PXE), Morcher2003, Wang2001 (0.4 in Caucasians), Gheduzzi2004, Struk2000, Hu2003a (34/32 in PXE, 80/204 in controls), Schulz2005b, SatoN2009 (studied amongst patients having AS but not full PXE, found in 33/108 AS alleles and 65/150 control alleles), RamsayM 2009 (!? Found in 3/10 black 1/10 white healthy south african person, do not mention if heteroz. or homoz.)	16
				16276345				Hendig 2005 (3/260 PXEp. - 2/200 control), Miksch2005 (no cosegregation with disease), Schulz2005a (7/674), Ramsay 2009 (uncorrect protein alteration	
c.2171G>A	p.R724K	P	m		E17	ICL6	-	reported)	17
				16276341				Hendig 2005 (3/260 PXEp. - 2/200 control), Miksch2005, Schulz2005a (7/674), RamsayM 2009 (Found in 2/10 blacksouth african pop. , do not mention if	
c.2175A>T	p.V725V	P	s		E17	ICL6		heteroz. or homoz.)	18
c.2224A>G	p.I742V	P	m	16276292	E17	ICL6		Hendig 2005 (3/260 PXEp. - 2/200 control), Miksch2005 (no cosegregation with disease), Schulz2005a (7/674) RamsayM 2009 (Found in 1/10 black south african pop. , do not mention if heteroz. or homoz.)	19
c.2400A>G	p.G800G	P	s	16272670	E18	ICL6		HapMap, RamsayM 2009 (Found in 2/10 black , 1/24 PXE patients in south african pop.do not mention if heteroz. or homoz.)	20
				16271409				Hendig 2005 (allele frequency data was not clear); LeSaux 2000; Hu 2003, HapMap, Miksch2005, Ringpfeil2001b, LeSaux2002 (6/48), Morcher2003, Wang2001 (0.08 in Caucasians), Gheduzzi2004, Schulz2006, Schulz2005b, RamsayM 2009 (Found in 3/24 PXE patient in south african pop., do not	
c.2490C>T	p.A830A	P	s		E19	ICL6	-	mention if heteroz. or homoz.)	21
				16271357				HapMap, RamsayM 2009 (Found in 3/10 black healthy . 1/24 PXE patient in	
c.2542A>G	p.M848V	P	m		E19	ICL6		south african pop, do not mention if heteroz. or homoz.)	22
c.2631C>A	p.T877T	P	s	16269803	E20	ICL6		Morcher2003(present in several non-PXE people)	23
c.2820T>G	p.R940R	P	s	16263678	E22	TMH12		Miksch2005	24
				16263663				Hendig 2005 (7/240); LeSaux 2002, HapMap, Morcher2003, RamsayM 2009 (Found in 2/10 black healthy, 1/10 white healthy, 1/24 PXE patient in south african pop, do not mention if heteroz. or	
c.2835C>T	p.P945P	P	s		E22	TMH12	-	homoz.)Miksch2005	25
				16263662				Hendig 2005(3/260 PXEp. - 7/200 control); Morcher 2003 (M), Miksch2005 (P, no cosegregation with disease), Shi2007, Hendig (P, because present in normal pop) vs Morcher (M), RamsayM 2009 (Found in 1/24 PXE patient in south african pop, do not mention if	
c.2836C>A	p.L946I	P	m		E22	TMH12		heteroz. or homoz.)	26
c.2904G>A	p.L968L	P	s	16263594	E22	ECL7	-	Hu2003b (est.freq.20%), Miksch2005	27
c.3190C>T	p.R1064W	P	m	16259596	E23	TMH14	+	Miksch2005 (does not cosegregate with disease), Morcher2003	28
				16251599				LeSaux2001 (23/244 of PXE chromosomes, 31/200 control chromosomes), HapMap, Miksch2005 (no segregation with disease+in LD with other PXE mut), LeSaux2002 (4/48 - no segregation with disease), Morcher2003, Wang2001 (0.04 in Africans; 0.06 in Chinese; 0.14 in Oji-Cree; 0.16 in South Asians; 0.17 in Inuit; 0.30 in Caucasians), Ringpfeil2000 (listed as mut.; 3/16), Gheduzzi2004, Hu2003a (G:0.3; A:0.7), Schulz2006, Germain2000 (24/124), Meyer2005, Schulz2005b (15/108 in PXE, 14/46 in relatives), SatoN2009 (found in healthy (48/300 control alleles) and AS (34/108 AS alleles) individuals in a study collecting patients with AS but not with the full diagnosys of PXE), QiaoliLi2011 (in association with the	
c.3803G>A	p.R1268Q	P	m		E27	ICL9	+	development of AS)	29
c.3978C>T	p.D1326D	P	s	16248793	E28	ICL9	+	Wang2001 (0.03 in Caucasians)	30
c.4254G>A	p.R1418R	P	s	16244584	E30	ICL9	-	Schulz2005a (6/266 in AAA; 2/244 in controls)	31
c.4305C>T	p.G1435G	P	s	16244533	E30	ICL9	-	Miksch2005	32

although not samesense alteration but unambigously prooved to be non disease causing

A	B	C	D	E	F	G	H	I	
sequence variation at DNA level	sequence variation at protein level	Mutation/ Polym.	type of mut. (exonic intronic)	genomic pos. according to ENSG00000091262.10; 2013. July	genomic region affected	protein region affected	C>T at 5'CpG	references (allele frequency data and comments)	
c.1-132C>T		P	i	16317423	5'flanking		+	Pulkkinen2001 (freq:0.953/0.047), Schulz2006 (freq:2/122 in PXE, 13/236 in controls)	1
c.191G>A	p.R64Q	?	m	16315534	E2	ICL1	+	LeSaux2001, also present in Psi2	2
				16313653				Miksch2005, RamsayM 2009 (!? Found in 1/10 white and 3/10 black healthy south african person, do not mention if heteroz. or homoz., in mixed South	
c.345+26C>T		P	i		I3		+	African pop.)	3
c.346-6G>A		P/M	i, pot. splice	16313545	I3		+	Miksch2005 (P), Pulkkinen2001 (P), Schulz2006 (M - affects splicing)	4
c.373G>A	p.E125K	M	m	16313512	E4	ICL2	+	Vanakker2008 (1/76)	5
c.473C>T	p.A158V	P?	m, pot.splice	16313412	E4	ECL3	+	Schulz2005b, could also be a frameshift, although they do not mention it	6
c.474+13G>A		P	i	16313398	I4		+	Miksch2005(intronic duplication)	7
c.496C>T	p.R166C	M	m	16308285	E5	ECL3	+	MartinL 2008 (found in a carrier, who has symptome, that closely mimic PXE, also reports on a PXE patient having c.496C>T, in compound heteroz. state with c.ABCC6del, but do not describes the patient in this paper, no controlls)	8
				16306059				Miksch2005, Pulkkinen2001, LeSaux2002 (2/48), Miksch2005, RamsayM 2009 (!? Found in 8/24 south african PXE patient, do not mention if heteroz. or	
c.645G>A	p.T215T	P	s		E6	ICL3	+	homoz.)	9
c.662+12C>T		P	i	16306030	I6		+	Miksch2005, Pulkkinen2001	10
				16297410				LeSaux2002 (found by chance, 2/48 in PXE, no controls!), Miksch2005(reports	
c.855C>T	p.T285T	P	s		E8	ICL3	+	this mutation as found in pseudogene1)	11
c.1091C>T	p.T364M	M	m	16295943	E9	TMH7	+	Vanakker2008	12
c.1144C>T	p.R382W	M	m	16295890	E9	ICL4	+	Pfendner 2007 (2/542)	13
c.1249G>A	p.V417M	P/M	m	16291967	E10	ICL4	+	RamsayM 2009 (!? Found in 5/24 PXE patient, do not mention if heteroz. or homoz., in mixed South African pop.)	14
c.1256G>A	p.R419Q	M?	m	16291960	E10	ICL4	+	Sato et al 2009 (associates with angioid streaks, in a study collecting patients with AS but not with the full diagnosys of PXE, 18/108 AS allele and 0/300 control allele)	15
c.1264G>A	p.E422K	M?	m	16291952	E10	ICL4	+	Sato et al 2009 (associates with angioid streaks ,AS, in a study collecting patients with AS but not with the full diagnosys of PXE, 1/108 AS allele and 0/300 control allele)	16
c.1312G>A	p.V438M	P	m	16291904	E10	TMH8	+	Sato et al 2009 (found in healthy individuals (1/300 control allele, 1 heterozygote) in a study collecting patients with AS but not with the full diagnosys of PXE)	17
c.1338+1548C>T		P	i	16290330	I10		+	HapMap	18
c.1460G>A	p.R487Q	M	m	16284196	E12	ICL5	+	Schulz2006	19
				16284104				Pfendner 2007 (2/542); Chassaing 2004; Gheduzzi 2004 (11/98); Meloni 2001; Miksch 2005 (1/170); Chassaing2005, Bergen2007, Ringpfeil2006,	
c.1552C>T	p.R518X	M	n		E12	ICL5	+	Schulz2006, Hu2003c, Hu2003b, Nitschke2012, Costrop 2010	20
				16284103				Pfendner 2007 (4/542); Chassaing 2004; Gheduzzi 2004 (5/98); LeSaux 2000; Miksch 2005 (4/170); Ringpfeil 2001, LeSaux 2001 (0.9% in EU, 1.4% in US); Uitto 2001, Chassaing2005, Bergen2007, LeSaux2002 (1/14), Hu2003c, Faria 2013, RamsayM 2009 (3/95 PXE alleles, 0.032 in mixed South	
c.1553G>A	p.R518Q	M	m		E12	ICL5	+	African pop.), Costrop 2010	21
				16283973				RamsayM 2009 (!? Found in 7/24 PXE patient, do not mention if heteroz. or homoz.) here they refer as it was descibed in Miksch 2005	
c.1635+48C>T		P?	i		I12		+	but it was not, there was most probably an errata in Miksch 2005	22
c.1636-240G>A		P	i	16283071	I12		+	HapMap	23
c.1636-112C>T		P	i	16282943	I12		+	HapMap	24
c.1779+381C>T		P	i	16282307	I13		+	HapMap	25
c.1798C>T	p.R600C	M	m	16281050	E14	ICL6	+	Pfendner 2007 (1/542), Costrop 2010	26
c.1868-57G>A		P	i	16278948	I14		+	Chassaing2004, Miksch2005, Gheduzzi2004	27
c.2247+302C>T		P	i	16275967	I17		+	HapMap	28
c.2248-552G>A		P	i	16273374	I17		+	HapMap	29
c.2263G>A	p.G755R	M	m	16272807	E18	ICL6 (Signature)	+	Pfendner 2007 (2/542)	30
				16272792				Pfendner 2007 (3/542); Schulz 2005a (1/108 in PXE patients); Gotting 2004;	
c.2278C>T	p.R760W	M	m		E18	ICL6 (Signature)	+	Miksch 2005 (1/170); Hendig 2005 (5/260), Schulz2006	31
c.2279G>A	p.R760Q	M	m	16272791	E18	ICL6 (Signature)	+	Vanakker2008 (2/76)	32
c.2293C>T	p.R765W	M	m	16272777	E18	ICL6 (Signature)	+	Vanakker2008 (1/76)	33
				16272776				Hendig 2005 (4/260); Hu 2003b (1/118); Hu 2004 (1/152); LeSaux 2002; Miksch 2005 (1/170); Schulz 2005a (3/108 in PXE patients); Gotting 2004, Pfendner 2007 (2/542); LeSaux 2001 (0.9% in EU, 0 in US), Chassaing2005, Bergen2007, Schulz2006, Hu2003c, LeBoulanger 2009 (most probably associated with GACI, uncorrectly reported mutation, most probably	
c.2294G>A	p.R765Q	M	m		E18	ICL6 (Signature)	+	R765Q)	34
c.2342C>T	p.A781V	M	m	16272728	E18	ICL6 (WalkerB)	+	Chassaing 2005, Bergen2007	35
c.2415+147C>T		P	i	16272508	I18		+	HapMap	36
c.2419C>T	p.R807W	M	m	16271480	E19	ICL6	+	Miksch 2005 (1/170) c.2419 is the 4th nucleotide posotion of e19 - possible splice mut?	37
c.2420G>A	p.R807Q	M	m	16271479	E19	ICL6	+	Miksch 2005 (1/170) c.2420 is the 5th nucleotide posotion of e19 - possible splice mut?, Chassaing2005, Bergen2007	38
c.2428G>A	p.V810M	M	m	16271471	E19	ICL6	+	Chassaing 2005; Gheduzzi 2004 (1/98), Bergen2007	39
c.2432C>T	p.T811M	M	m	16271467	E19	ICL6	+	Pfendner 2007 (1/542); Ringpfeil 2006	40
c.2666+499C>T		P	i	16269269	I20		+	HapMap	41
c.266+900C>T		P	i	16268868	I20		+	HapMap	42
c.2787+30G>A		P	i	16267111	I21		+	LeSaux2002 (2/48)	43
c.2848G>A	p.A950T	M	m	16263650	E22	TMH12	+	Pfendner 2007 (1/542)	44
c.2995+142C>T		P	i	16263361	I22		+	HapMap	45
c.2996-637G>A		P	i	16260427	I22		+	HapMap	46
c.2996-552G>A		P	i	16260342	I22		+	HapMap	47
c.3088C>T	p.R1030X	M	n	16259698	E23	ICL7	+	LeSaux 2001 (1/202), Gheduzzi 2004 (5/98); Chassaing 2005, Bergen2007, LeSaux2002, Hu2003c	48
c.3190C>T	p.R1064W	P	m	16259596	E23	TMH14	+	Miksch2005 (does not cosegregate with disease), Morcher2003	49
c.3308+531C>T		P	i	16258949	I23		+	HapMap	50
c.3307-1124C>T		P	i	16258173	I23		+	HapMap	51
c.3340C>T	p.R1114C	M	m	16257016	E24	ICL8	+	Gheduzzi 2004 (1/98); Miksch 2005 (1/170); Schulz 2005a (1/108 in PXE patients); Gotting 2004; Klein 2001, Chassaing2005, Bergen2007, Schulz2006, Schulz2005b (1/108), Nitschke 2012	52
c.3341G>A	p.R1114H	M	m	16257015	E24	ICL8	+	Hu2003b (1/118), Götting2004(1/128), Chassaing2005, Bergen2007, Hu2004 (1/152), Schulz2005a (1/108 in PXE patients), Schulz2006, Schulz2005b (1/108)	53
c.3362C>T	p.S1121L	M	m	16256994	E24	ICL8	+	Miksch 2005 (3/170)	54
c.3389C>T	p.T1130M	M	m	16256967	E24	ICL8	+	Chassaing 2004; Gheduzzi 2004 (2/98); Hu 2003; Hu 2004;Miksch 2005; Ringpfeil 2000; Schulz 2005a (2/108 in PXE patients); Gotting 2004 (2/128); Klein 2001, Pfendner 2007, Chassaing2005, Bergen2007, Morcher2003, Schulz2006, Shi2007 (1/32), Schulz2005b (2/108), Martin L2008	55
c.3412C>T	p.R1138W	M	m	16256944	E24	ICL8	+	Ringpfeil 2000 (1/16); Miksch 2005 (1/170), Uitto2001, LeSaux2001, Chassaing2005, Bergen2007, Ringpfeil2006, Schulz2006, Hu2003c, Ringpfeil2001, Shi2007(2/32), HesseRJ 2010	56
c.3413G>A	p.R1138Q	M	m	16256943	E24	ICL8	+	LeSaux 2001 (0.9% in EU, 1.4% in US); Miksch 2005 (3/170); Ringpfeil 2000 (1/16), LeSaux2000, Uitto2001, Chassaing 2005; Gheduzzi 2004 (1/98); Chassaing 2004, Bergen2007, LeSaux2002 (3/34 in Afrikaner, 0 in other SA), Schulz2005a (2/108 in PXE patients), Schulz2006, Fabre2005, Hu2003c, Ringpfeil2001, Shi2007 (3/32), Schulz2005b (2/108), RamsayM 2009 (6/95 PXE alleles, 0.063 in mixed South African pop.)	57

								Pfendner 2007; Ridnip 2004; Falkinen 2001; Bergen 2000; Chassaing 2004; Gheduzzi 2004 (36/98); Hu 2003b (22/118); Hu 2004 (35/152); LeSaux 2000; Miksch 2005 (45/170); Ringpfeil 2000 (4/16); Ringpfeil 2001b; Schulz 2005a (28/108 in PXE patients, 9/46 in PXE relatives); Struk 2000; Gotting 2004 (33/128 PXE patients; 1/1820 healthy donors); Klein 2001, LeSaux 2001 (18.8% in EU, 4.1% in US), Uitto 2001, Germain2000, Hendig2005 (38/260 in PXE+related, 1/200 in healthy donors); Trip2002(22/2996, all in heterozygous form), Cai2001, Chassaing2005, Bergen2007, LeSaux2002 (2/34 in Afrikaner, 2/14 in other SA), Audo2007, Ringpfeil2006, Hu2003a, Schulz2006, Hu2003c, Ringpfeil2001, Bowen2007, Shi2007 (5/32), Schulz2005b (28/108 in PXE, 9/46 in relatives), Drera2007 (experimentally validated: in trans with G1133C), Faria 2013, RamsayM 2009 (9/95 PXE alleles, 0.095 in	
				16256935					
c.3421C>T	p.R1141X	M	n		E24	ICL8	+	mixed South African pop.), LaRusso 2010 (32/140), Nitschke 2012 Chassaing 2004; Meloni 2001; Miksch 2005 (9/170); Ringpfeil 2001b; Struk 2000, Pfendner 2007, Uitto2001, LeSaux2001, Chassaing2005,	58
				16256866					
c.3490C>T	p.R1164X	M	n		E24	ICL8	+	Bergen2007, Wang2001, Hu2003c, Ringpfeil2001, Shi2007 (6/32)	59
c.3491G>A	p.R1164Q	M	m	16256865	E24	ICL8	+	Pfendner 2007; Miksch 2005 (1/170), Ringpfeil2006	60
				16253413				Hu 2003; Hu 2004; Miksch 2005 (2/170); Noji 2004; Pfendner 2007,	
c.3661C>T	p.R1221C	M	m		E26	ICL9	+	Bergen2007, Costrop 2010	61
c.3662G>A	p.R1221H	M	m	16253412	E26	ICL9	+	Cotton 1998, Pfendner 2007, Nitschke 2012	62
c3703C>T	p.R1235W	M	m	16253371	E26	ICL9	+	Miksch 2005 (2/170)	63
c.3736-502C>T		P	i	16252168	I26		+	HapMap	64
c.3787G>A	p.G1263R	M	m	16251615	E27	ICL9	+	Vanakker2008 (1/87)	65
				16251599				LeSaux2001 (23/244 of PXE chromosomes, 31/200 control chromosomes), HapMap, Miksch2005 (no segregation with disease+in LD with other PXE mut), LeSaux2002 (4/48 - no segregation with disease), Morcher2003, Wang2001 (0.04 in Africans; 0.06 in Chinese; 0.14 in Oji-Cree; 0.16 in South Asians; 0.17 in Inuit; 0.30 in Caucasians), Ringpfeil2000 (listed as mut.; 3/16), Gheduzzi2004, Hu2003a (G:0.3; A:0.7), Schulz2006, Germain2000 (24/124), Meyer2005, Schulz2005b (15/108 in PXE, 14/46 in relatives), SatoN2009 (found in healthy (48/300 control alleles) and AS (34/108 AS alleles) individuals in a study collecting patients with AS but not with the full diagnosys of PXE), QiaoliLi2011 (in association with the	
c.3803G>A	p.R1268Q	P	m		E27	ICL9	+	development of AS)	66
c.3823C>T	p.R1275X	M	n	16251579	E27	ICL9	+	Chassaing 2005; Gheduzzi 2004 (3/98), Bergen2007	67
c.3871G>A	p.A1291T	?	m	16251531	E27	ICL9	+	Hefferon2007	68
	p.A1295fsX1321			16248894				Miksch 2005 (1/170) gain of ss mutation which causes frame shift and premature stop; LeSaux2002 (only reported as a polymorphism, but 1/48 PXE alleles and	
c.3883-6G>A	gain of ss	M/P	i, pot. splice		I27		+	they have no controls!), Schulz2006 (M, altered splicing)	69
				16248867				Pfendner 2007; Hu 2003c; Hu 2004 (1/152); LeSaux 2001 (0 in EU, 5.4% in US); Miksch 2005 (1/170), Chassaing2005, Bergen2007, Shi2007 (3/32),	
c.3904G>A	p.G1302R	M	m		E28	ICL9 (WalkerA)	+	Ramsay M2009	70
				16248831				LeSaux 2000 (located at WalkerA in NBF2); LeSaux 2001 (0 in EU, 4.1% in US); Schulz 2005a (1/108 in PXE); Pfendner 2007, Uitto2001, Chassaing2005, Bergen2007, Schulz2006, Hu2003c, Ringpfeil2001, Schulz2005b (1/108), in LD with 4042-30C>T? (Shi2007), Nitschke	
c.3940C>T	p.R1314W	M	m		E28	ICL9	+	2012	71
				16248830				Pfendner 2007; Klein 2001, LeSaux 2001 (1/116 in EU); Miksch 2005 (1/170), Chassaing2005, Bergen2007, Schulz2005a (1/108 in PXE, 1/46 in PXE relatives), Schulz2006, Hu2003c, Schulz2005b (1/108 in	
c.3941G>A	p.R1314Q	M	m		E28	ICL9	+	PXE, 1/46 in relatives)	72
c.3978C>T	p.D1326D	P	s	16248793	E28	ICL9	+	Wang2001 (0.03 in Caucasians)	73
				16248756				Pfendner 2007; Gheduzzi 2004 (3/98); LeSaux 2001 (0 in EU, 2.7 in US); Miksch 2005 (11/170); Struk 2000, Uitto2001, Chassaing2005, Bergen2007, LeSaux 2002 (18/34 in Afrikaner, 0 in other SA), Hu2003c, Ringpfeil2001, Shi2007 (2/32), Faria 2013, RamsayM 2009 (39/95 PXE alleles, 0.411 in mixed South African pop.), LaRUsso2010 (11.5% in mediterraneas, 9.1% of	
c.4015C>T	p.R1339C	M	m		E28	ICL9	+	scandinavians of mutated alleles)	74
c.4016G>A	p.R1339H	M	m	16248755	E28	ICL9	+	Pfendner 2007;	75
c.4041+49C>T		P	i	16248681	I28		+	Miksch2005	76
				16248681				LeSaux2000, HapMap, Miksch2005, LeSaux2002 (18/48), Schulz2005a (?), Bowen2007, Shi2007 (3/32), in LD with 3940C>T?	
c.4042-30C>T		P	i		I28		+	(Shi2007)	77
c.4069C>T	p.R1357W	M	m	16248624	E29	ICL9	+	Miksch 2005 (1/170), Noji2004	78
c.4081G>A	p.D1361N	M	m	16248612	E29	ICL9	+	LeSaux 2001 (1/116 in EU), Chassaing2005, Bergen2007, Hu2003c	79
c.4192C>T	p.R1398X	M	n	16248501	E29	ICL9	+	LeSaux 2001 (1/116 in EU), Pfendner 2007, Chassaing2005,	80
				16248495				Chassaing 2004, Chassaing2005, Gheduzzi2004 (4/98), Bergen2007, Faria	
c.4198G>A	p.E1400K	M	m		E29	ICL9	+	2013,	81
c.4208+9G>A		P	i	16248476	I29		+	Miksch2005, Schulz2005a (5/266 in AAA)	82
c.4209-1100C>T		P	i	16245729	I29		+	HapMap	83
c.4253G>A	p.R1418Q	M	m	16244585	E30	ICL9	+	Meyer2005	84
c.4279G>A	p.E1427K	M?	m	16244559	E30	ICL9 (WalkerB)	+	Sato et al 2009 (associates with angioid streaks, in a study collecting patients with AS but not with the full diagnosys of PXE, 1/108 AS allele and 0/300 control allele)	85
c.4404-81C>T		P	i	16244179	I30		+	Hendiq (1/260)	86
c.4441G>A	p.G1481S	M	m	16244061	E31	ICL9	+	Pfendner 2007	87
c.4448C>T	p.P1483L	?	m	16244054	E31	ICL9	+	Hefferon2007	88
c.4512+17G>A		P	i	16243973	3'UTR		+	LeSaux2000, Miksch2005, LeSaux2002 (2/48)	89
c.4512+38G>A		P	i	16243952	3'UTR		+	Miksch2005	90